Extending the Shelf life of a Value-added Meat Product: the Influence of Myoglobin Oxidation in Fresh Pork sausages

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ABSTRACT

The purpose of this study was to assess factors that can influence the colour stability of fresh sausage products using a pork patty model system over a typical distribution and display period. Fresh sausage is usually sold in raw; and it should have minimum 7.5% meat protein and 9% total protein. Losses of meat quality were evidenced through the discolouration of meat, depletion of endogenous antioxidant activities, proliferation of spoilage microorganisms, and reduction in the meat redox potential.

Both ground pork and fresh pork patties were made from pork picnic shoulder. In the first study, the quality of both ground pork patties and fresh pork sausage patties decreased over time during storage at 4°C. The fresh sausages contained ingredients that could prolong their shelf life. The activities of these antioxidant enzymes in both ground pork and fresh sausage were depleted by day 5 of the display period. Ground pork, however, had significantly (p<0.05) higher activity of catalase, glutathione peroxidase (GSHPx), superoxide dismutase (SOD) and total antioxidant activity (TEAC) than fresh sausage due to the effect of the salt. Moreover, there was no significant treatment effect on microbial numbers but there was a significant (p<0.05) elevation of microbial colony forming units by day 5 of the display period. The elevation of microbial numbers by the end of the display period was consistent with the drop in redox potential that was measured near the surface of the patties at the end of the incubation period.

In the second study, there was no synergistic effect ($p<0.05$) between sodium erythorbate and lemon juice powder that were used to enhance colour stability during storage and display in terms of antioxidant activity, colour and microbiological profile. The addition of sodium erythorbate alone, however, had a significant effect ($p<0.05$) on catalase activity and a* values. In other words, this catalase activity was more effective in protecting against oxidation with the addition of sodium erythorbate so that the redness of the fresh sausages (a*values) was preserved. Furthermore, the combined addition of sodium erythorbate and lemon juice powder did not have any antimicrobial activity because there was no significant difference in total microbial counts (*Brochothrix thermosphacta* count and lactic acid bacteria) following the addition of those ingredients. The measurement of redox potential near the surface of fresh pork patties could not be conclusively correlated with the addition of non-meat ingredients or microbiological activity. However, the measurement of redox potential in the middle of fresh pork patties showed that the addition of sodium erythorbate lowered the redox of the fresh sausage B (0.05% sodium erythorbate) and D (0.25% lemon juice powder and 0.05% sodium erythorbate) compared to fresh sausage A (0.00% lemon juice powder and 0.00% sodium erythorbate).

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1.0 Introduction

Meat is a perishable food item, and meat spoilage is a significant concern for the meat industry. The spoilage of meat and meat products is highly correlated with shelf-life, which is the total time that a meat product remains desirable enough to be saleable (Smith and Lesser, 1982). In addition, meat colour is regarded as the first limiting factor in shelf-life because customers tend to use meat colour as the chief indication of freshness. Autoxidation of oxymyoglobin, the desirable oxygenated pigment of muscle tissue in meat and meat products, yields metmyoglobin, which has an undesirable brown and sometimes grey colour. Though misguided, consumers often equate this grey colour as an indication of meat spoilage.

Muscle tissue has endogenous antioxidant mechanisms to help control oxidation *in vivo* and these antioxidant activities continue to operate for a limited period *post mortem* (Monahan, 2000). These endogenous antioxidants are believed to be chiefly responsible for the shelf-life stability of meat colour in meat and meat products *post mortem* (Gutteridge and Halliwell, 1994). Important endogenous antioxidant enzymes include superoxide dismutase (SOD), glutathione peroxidase (GSHPx) and catalase (CAT). SOD and CAT are coupled enzymes: SOD catalyzes the conversion of the superoxide anion to hydrogen peroxide whereas CAT catalyzes the conversion of hydrogen peroxide to water and oxygen. The glutathione system (*i.e.*, glutathione,

glutathione peroxidase and glutathione reductase) is a key defense system against the build up of H_2O_2 and other peroxides (Gatellier *et al*., 2004). Meat processing and the addition of non-meat ingredients could affect the stability of these enzymes and thereby affect the stability of meat colour. The efficacy of endogenous antioxidant constituents in meat and meat products at combating oxidative processes diminishes with increasing time *post mortem* (storage) and may possibly be employed to predict the shelf-life of meat patty colour.

The overall redox potential of pork patties is expected to decrease over time and this may be due to changes in the activities of endogenous enzymes in the meat model system. The redox potential of meat is influenced by intrinsic factors related to the product, as well as extrinsic factors imposed during meat processing (Rödel and Scheuer, 2000). Both factors play a role in defining the quality of the final meat product.

The point of spoilage may be defined by a certain maximum acceptable bacterial concentration, so the product's shelf-life depends on the numbers and types of microorganisms initially present and their subsequent growth during storage. Moreover, the growth of microorganisms in the meat products could alter the redox conditions. Thus, the redox potential may function as an indicator of the microbial stability of the product, and also be used to assess the process of decomposition of fresh meat during storage to determine the overall meat quality deterioration over time.

The aim of this thesis research is to assess factors (time and non-meat ingredients) that can influence the colour stability of fresh sausage products using a pork patty model system during storage. Endogenous antioxidant activities, the degree of microbial proliferation and the influence of non-meat ingredients in patty formulations

will be related to the redox potential of the pork patties during refrigerated storage to assess relationships between redox potential as an indicator of spoilage and the shelf-life colour stability of the pork patties.

The objectives of this study were:

- To determine the effect of time on the stability of pork patties in terms of their chemical (redox potential and antioxidant enzyme activities), colour and microbiological (types and numbers of bacteria) characteristics during storage.
- To assess the effect of non-meat ingredients on the stability of pork patties in terms of their chemical (redox potential and antioxidant enzyme activities), colour and microbiological (types and numbers of bacteria) characteristics over time.

2.0 Literature Review

2.1 Meat quality

Meat quality is defined by the consumer's buying habits, but consumers preferences are not always related to quality grades; therefore, quality must be supported by a specific concrete benefit for the consumer. Different consumers will have varying definitions of "product quality," and their perception will depend on their beliefs and habits which are strongly dependent on their cultural traditions, on their age and on their education. Perceived quality is not only dependent on the consumer but also on the context, such as the circumstances in which food and the consumer interact. Schuts (1988) argues that a critical determinant of food acceptability is its perceived appropriateness in a certain situations. Therefore, a given product can be perceived to have more or less quality, depending on its usage, which in turn can motivate the consumer to buy that particular product.

Meat quality has different components, as indicated in Table 2.1. Meat quality can also be differentiated into either functional quality or conformance quality. The former refers to the desirable attributes in a product and the latter refers to the product's ability to meet the customer's expectation (Lawrie, 1991). Among all these components of quality, customers purchasing meats, in practice, judge quality firstly from the perspective of appearance, most notably colour, and this has been shown to strongly influence their purchase decision.

Table 2.1: Major meat quality categories. Adapted from Smulder and van Laack, 1992

Categories	Factors
Hygiene	Spoilage and pathogenic microorganisms
	Intrinsic factors: A_w, Eh and pH
	Contaminants/residues
Food physiology	Chemical composition
	Nutritive value
Technology	Fat, connective tissue-, tendon-content
	Consistency and structure
	Water-binding properties
Sensory	Appearance: colour (stability), shape and size
	Eating quality: tenderness, water-holding capacity (juiciness) and flavour

2.2 Meat shelf-life

The general definition of shelf-life is the length of time in which a product can be stored under specified conditions with a tolerable loss in "quality" and still be acceptable for consumption (Lawrie, 1991). Of course, "quality" and "acceptable" are subjective terms. The shelf-life of a product is critical in determining both its quality and profitability.

Meat colour is regarded as the first limiting factor in shelf-life because customers often use meat colour as indication of freshness. The oxidation of oxymyoglobin in meat tissues will form metmyoglobin which has brown colour that the customers consider an indication of meat spoilage. On the other hand, the bright red colour of oxymyoglobin is more desirable for customers and they use this colour as an indication of meat freshness. Therefore, it is very desirable to delay pigment oxidation and enhance the reduction of

oxidized pigment in order to maximize acceptable fresh meat colour so that the meat can have longer shelf life (Faustman and Cassens, 1990a).

2.3 The nature of colour

Light waves, when striking an object, can have varying degrees of three fates: it can pass through the object, become absorbed by the object, or be reflected by the object. The wavelengths that are reflected back from the object are important for perceived colour. Furthermore, colour that can be visually detected has several attributes: hue, chroma and value. Hue is the wavelength of light radiation and is perceived as yellow, green, blue or red colour. Chroma is the intensity of the colour and it is associated with white light that is mixed with colour. Value indicates the brightness that is associated with the reflectance of the colour.

2.3.1 Meat colour

Pigments which can absorb certain wavelengths of light are responsible for meat colour (Hedrick et al., 1989), but there are many factors that influence how our eyes perceive these colours, including the structure and texture of the muscles (Warris, 2000). Myoglobin is the primary pigment (80%-90%) that responsible for meat colour (Hedrick et al., 1989). Other haem proteins, such as hemoglobin and cytochrome C, also contribute to a lesser extent to meat colour (Mancini and Hunt, 2005; Lindahl, 2005). Meat colour depends on pigment content, ultimate pH, rate of pH decline *post mortem*, as well as the physical characteristics of the muscle (Reneree, 2000).

2.3.2 Colour intensity

Color intensity in meat is dependent on the concentration of myoglobin, a factor which varies with species, sex, muscle type and physical activity (Hedrick *et al*., 1989). In general, beef and lamb have more myoglobin than pork, veal, fish and poultry (Table 2.2). Males usually have muscle that contains more myoglobin than females at comparable ages. Mature animals also usually have more myoglobin than immature animals; for example, veal has a myoglobin content of 1-3 mg/g wet tissue, whereas mature beef has 16-20 mg of myoglobin per gram of wet tissue (Hedrick *et al*., 1989).

Within an animal, different muscles can have different amount of myoglobin. Oxidative muscles (red) have more mitochondria and a higher concentration of myoglobin in their sarcoplasm. This type of muscle generates ATP through oxidative metabolism. On the other hand, glycolytic muscles (white) contain small amount of myoglobin and they perform glycolysis to generate ATP. White muscles are slow-contracting muscles that are usually characteristic of postural muscles, whereas red muscles are fast-contracting muscles that play important role in rapid and intermittent movement (Warris, 2000). Therefore, different cuts of meat contain varying proportions of different muscles types and this determines the macroscopic colour of the meat (Warris, 2000). For example, muscles with major function in movement tend to have more red muscles than white muscles and therefore have more intense red colour (Table 2.3).

Table 2.2: Myoglobin in muscles of several species or breeds. Reproduced from Young and West, 2001

Species	Approximate concentration (mg/g)
Cattle (breed unstated)[a]	2 – 5
Sheep (Dorset)[b]	3 – 7
Pig (Hampshire)	3 – 6
Wild pig	Higher than for domestic pigs
Chicken	0.1 – 5
Tuna[c]	0.5 – 1
Human[a]	4.4 - 5.2
Whale (*Hyperoodon rostratus*)	60
Dolphins	50 – 72
Seal (*Phoca vitulina*)	80

[a] For a range of muscles
[b] *Longissimus*
[c] Light muscles

Table 2.3: Concentration of myoglobin (mg/g) within muscles of different species. Reproduced from Young and West, 2001

	Species			
Muscle	Cattle	Pig (Hampshire)	Tuna	
Lonngisimus	3.48	2.94	Light meat	0.7
Psoas major	3.71	6.37	Lateral line muscle	20
Glueteus medius	4.11			
Semimembranosus (outer)	3.91	4.05	**Chicken**:	
Semimembranosus (inner)	3.56		Pectoralis	0.1
Semintendinosus (outer)	2.97		Vastus lateralis	2.8
Semitendinosus (inner)	1.95		Vastus intermedius	5.0
Biceps femoris		5.06	Biceps femoris	0.7
Rectus femoris		5.66	Rectus femoris	2.5
Diaphragma	~7		Gizzard	19

2.4 Structure of myoglobin

Myoglobin is the pigment (80-90%) of well-bled muscle tissue, and is chiefly responsible for meat colour (Hedrick *et al*, 1989). Other haemoproteins, such as haemoglobin and cytochrome C, also contribute to meat colour, but to a much lesser extent (Mancini and Hunt, 2005; Lindahl, 2005). Haemoglobin is basically a tetramer of myoglobin, and is a component of red blood cells. Moreover, cytochromes are haem proteins that function as part of electron transport chain in living cells.

Myoglobin is found in the sarcoplasmic fraction of muscles (Govindarajan, 1973). It carries oxygen from capillaries to the intracellular locations of muscle tissue and facilitates muscle movement (Renerre, 2000). Myoglobin quantity varies with species, sex, muscle and physical activity (Hedrick *et al*., 1989). Myoglobin is a monomeric, globular haemoprotein with a molecular weight of 17000 Da (Renerre, 2000), and consists of a protein portion that is known as globin and a non-protein portion, which is referred to as a haem ring (*i.e.*, a protoporphyrin). The globular protein consists of 153 amino acids that are 80% in the alpha-helix conformation with the interior of the protein comprised of mostly the non-polar residues Leu, Val, Met and Phe. Most of the polar residues are located at the myoglobin surface, and the 2 histidines located near at haem binding site are the only polar residues that are located in the core of the protein.

The haem moiety of myoglobin has a planar structure on account of its tetrapyrrole structure with an iron atom at its centre. In the ferrous state, the iron atom has the capability to form six bonds (some covalent, some coordinate). Four of these

bonds have an iron atom attached to the nitrogen of the pyrole residues. The fifth bond links the iron atom to the proximal histidine-93 residue of globin (apoprotein) and the sixth one offers a coordination site that is available for binding a variety of the ligands (Livingston and Brown, 1982). There is also a second histidine residue, which is called the distal histidine, near the haem but it is not bonded to it (Renerre, 2000). This distal histidine protects the haem against oxidation and also blocks carbon monoxide from binding to iron while allowing oxygen to bind iron easily.

2.4.1 Myoglobin species

Meat colour predominantly depends on the chemical state of the iron present in the haem ring, but also depends on the type of ligand attachment on the sixth bond of iron (Lindahl, 2005). Myoglobin can be found in three different species: deoxymyoglobin (myoglobin), oxymyoglobin and metmyoglobin, as shown in Figure 2.1. Besides these three major species, myoglobins can complex with NO to form nitric oxide myoglobin ($Mb^{2+}NO$), and can also bind carbon monoxide to form carboxymyoglobin ($Mb^{2+}CO$). $Mb^{2+}NO$ is the basis of cured meat technology and it is responsible for the bright pink colour in cured meat. Myoglobin has greater affinity for carbon monoxide (CO) than oxygen and carboxymyoglobin ($Mb^{2+}CO$) will also result in pink meat colour.

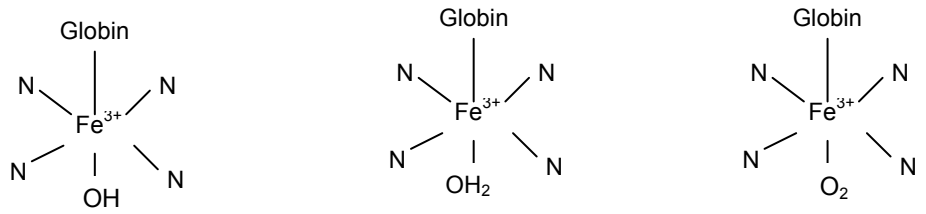


Figure 2.1: The iron chemical state and ligand attachment on the 6th coordination determine pigment species and colour (Adapted from Hedrick *et al*., 1989)

2.4.2 Reactions of myoglobin with oxygen

The haem iron may exist in a reduced ferrous or oxidized ferric form. The sixth coordination of the iron in the myoglobin reversibly binds oxygen. Ferrous haem iron, which lacks a sixth ligand, is called deoxymyoglobin, or myoglobin. It has a purple colour, and the oxygenation of deoxymyoglobin which is in the ferrous state forms oxymyoglobin which has bright red colour (Reaction 2.1) (Mancini and Hunt, 2005; Renerre, 2000). Oxymyoglobin is formed as soon as meat exposed to air but its stability depends on a continuing supply of oxygen which is also used by enzymes involved in the oxidative metabolism of mitochondria (Hedrick *et al*., 1989).

Mb^{2+} (purple-red) + O_2 → $Mb^{2+}O_2$ (bright red) (Reaction 2.1)

It is important to remember that in reaction 2.1 the iron is in the reduced state (Fe^{2+}) and the only thing changing is the sixth iron-binding site. The sixth coordination site is not exclusively for oxygen; however, there are some restrictions for what molecules that can bind in order to make a stable myoglobin complex. The cleft in sixth

coordination is small so only small molecules with low energy state can form stable complex with myoglobin. Generally, there are only 3 ligands that are important for meat colour: oxygen, nitric oxide and carbon monoxide (Reneree, 2000).

Both deoxymyoglobin and oxymyoglobin readily oxidize to produce the brown-coloured metmyoglobin; this reaction is reversible and oxygen partial pressure-dependent (Figure 2.2). The oxidized iron in metmyoglobin, Fe^{3+}, cannot bind oxygen in its sixth coordination; instead, it binds water. Metmyoglobin is responsible for the discoloration of fresh meat during storage (Tang *et al*., 2003; Lindahl, 2005). Metmyoglobin's brown colour is noticeable when 60% of myoglobin exists in this form (Lawrie, 1991).

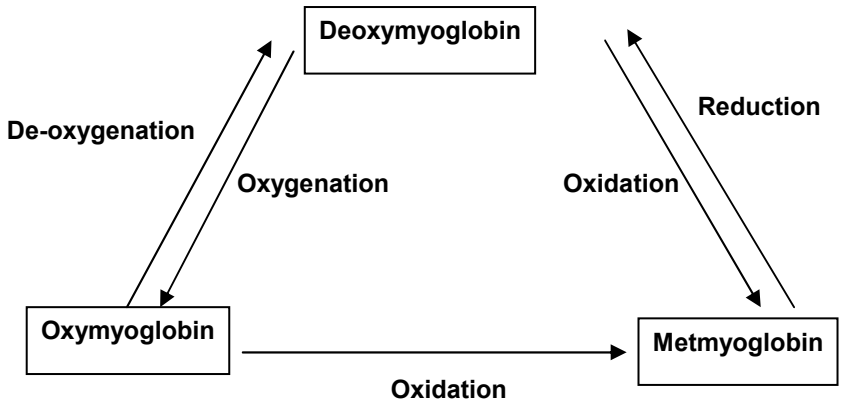


Figure 2.2: Flow chart for reduced myoglobin to oxymyoglobin to metmyoglobin formation

2.5 Biochemical factors affecting metmyoglobin formation

The formation of metmyoglobin in meat is mainly affected by the oxygen consumption rate, myoglobin autoxidation rate and metmyoglobin reduction (Renerre, 2000).

2.5.1 Oxygen consumption rate

The bright red colour of meat depends on how deep molecular oxygen can penetrate into meat tissues; the depth of this penetration depends on the rate of oxygen diffusion, the rate of oxygen consumption, and the partial pressure of oxygen (O'Keeffe and Hood, 1982; Renerre, 2000). The oxygen consumption rate (OCR) decreases with time due to the depletion of substrate coenzymes and the degradation of enzymes involved in mitochondrial respiration (O'Keeffe and Hood, 1982). Mitochondria retain their oxidative capacity within intact tissue for 6 days at 4°C; moreover, Bendall and Taylor (1972) found that the oxygen consumption rate *post mortem* muscle decreased exponentially during 2 to 6 days storage at 2°C. As a consequence, oxygen can penetrate even further into the depth of the meat with time (Renerre, 2000). The balance between metabolic oxygen consumption and oxygen binding by myoglobin changes over time (Young and West, 2001) and differs among muscles. Muscles, that have higher rates of oxygen consumption, become discoloured more rapidly.

2.5.2 Myoglobin autoxidation

Myoglobin autoxidation involves the non-enzymatic spontaneous oxidation of myoglobin, which produces metmyoglobin in fresh meat (Renerre *et al*., 1996). The autoxidation rate can be influenced by oxygen partial pressure, temperature, pH, the presence of non-meat ingredients, oxidation and reduction initiators, and the amount of catalytic heavy metals. Temperature is the most important factor because the production of metmyoglobin can be enhanced when the globin part of myoglobin is denatured at high temperature (Lawrie, 1991). Moreover, as temperature increases, mitochondrial respiratory enzymes are more active (high OCR) in scavenging oxygen, which in turn leads to low oxygen tension that favours autoxidation of myoglobin (Reneree, 2000). The autoxidation rate will decrease by ~40 fold as the temperature decreases from 22°C to -2°C at any pH (Brown and Mebine, 1969).

pH 5.5 has the highest rate of myoglobin oxidation, and that this rate continues to decrease rapidly until pH 6.0 at which point it slowly levels out (Brown and Mebine, 1969). Myoglobin is denatured and the enzymatic reduction of metmyoglobin is inactivated at low pH. Therefore, at low pH meat discolouration rate is faster than at the higher ultimate pH. The maximum rate of metmyoglobin conversion is at a partial pressure 1.5 mm of mercury when 50% of myoglobin is saturated with oxygen; this then declines and levels out at around 40 mm in normal muscle. In order to minimize metmyoglobin formation in fresh meat, oxygen must be totally excluded from the packaging environment or present at saturating levels.

2.5.3 Metmyoglobin reduction

Metmyoglobin may be converted back to its physiologically-active form by a process called reduction, and this reduction is facilitated by enzymes known as metmyoglobin reductases. Metmyoglobin is stable and is reconverted to deoxymyoglobin slowly by metmyoglobin reduction enzymes (Ledward, 1985). It is very important to maintain myoglobin in its reduced state in order for myoglobin to perform its physiological role because only in the reduced form can myoglobin bind oxygen (Reneree, 2000) and produce the desirable red colour. *Post mortem*, metmyoglobin reduction activity decreases over time because the fall in tissue pH, the degradation of substrates and cofactors, and the loss of mitochondrial structural functionality

Haemoglobin (Hb) and myoglobin (Mb) share many physiological and biochemical properties. Both haemoglobin and myoglobin undergo oxidation to form physiologically inactive methaemoglobin (MetHb) and metmyoglobin (MetMb), respectively. The fact that only 0.5% of Hb is in the oxidized form suggests that there is a mechanism that reduces MetHb back to Hb (Van Slyke *et al*., 1946). Mb is more prone to oxidation than Hb and it is likely that Mb also has an analogous reduction mechanism as in Hb.

It has been reported that there are two different enzymes responsible for metmyoglobin reduction: DT-diaphorase [NADPH-quinone oxireductase] and MetMb reductase [NADH-cytochrome $\boldsymbol{b}_5$ oxireductase] (Hagler *et al*., 1979). Non-specific diaphores, which are located in the cytosol, are also capable of reducing both methaemoglobin and metmyoglobin at the expense of NADH, but at a slower rate than

the more-specific reductase (Leroux and Kaplan, 1975). On the other hand, NADH – cytochrome $\boldsymbol{b}_5$ oxireductase is the best-characterized enzyme involved in the metmyoglobin reduction system.

Arihara *et al*. (1989) successfully isolated MetMb reductase from bovine cardiac muscle and they stated that it had optimum pH of 6.5, an isoelectric point of 5.6-5.8 and a molecular weight of 33,000 daltons. The main function of this enzyme is to transfer the two electrons from NADH to two molecules of cytochrome $\boldsymbol{b}_5$ (Reaction 2.2). Then, the reduced cytochrome $\boldsymbol{b}_5$ transfer electrons to a variety of acceptors including MetHb or MetMb.

NADH + 2ferricytochrome $\boldsymbol{b}_5$ <=> NAD^+ + 2ferrocytochrome $\boldsymbol{b}_5$ (Reaction 2.2)
(Cofactor(s): Fad)

Cytochrome is a membrane-bound haemoprotein that carries out electron transport. Several kinds of cytochromes exist and they can be distinguished by spectroscopy, the exact structure of the haem group, inhibitor sensitivity, and reduction potential. Mammalian liver cytochrome $\boldsymbol{b}_5$ in the reduced state has 3 maximum absorbance peaks at 556 nm, 526 nm and 423 nm (Hagihara *et al*., 1975). Moreover, in the oxidized form, cytochrome $\boldsymbol{b}_5$ has an absorption maximum at 413 nm (Hagihara *et al*., 1975). NADH – cytochrome $\boldsymbol{b}_5$ reductase requires either cytochrome $\boldsymbol{b}_5$ or outer membrane cytochrome ***b*** (OM cytochrome ***b***) as electron transfer mediators for MetMb reduction (Arihara *et al*., 1995). Arihara *et al*. (1995) proposed 2 pathways for MetMb reduction. First, NADH– cytochrome $\boldsymbol{b}_5$ reductase uses OM cytochrome ***b*** at the mitochondrial surface to prevent MetMb accumulation in muscle. NADH – cytochrome $\boldsymbol{b}_5$ reductase also uses

cytochrome $\boldsymbol{b}_5$ to reduce MetMb to some extent at the sarcoplasmic reticulum (Arihara *et al*., 1995).

2.6. Meat colour from slaughter to display

At slaughter, the vascular system ceases to deliver oxygen to muscles so that mitochondria use oxygen from oxymyoglobin to sustain respiration. At this point, the muscle pH is still in the normal range as in live and rested muscle (pH 7.0) so that the meat colour is purple-red due to more deoxymyoglobin in the meat (Young and West, 2001). Over time, glycogen is broken down through glycolysis to provide ATP for muscles. Glycolysis produces lactic acid which reduces the meat pH. At this time, unstressed well-fed animals can have ultimate or final pH of around 5.5. As the pH drops, the myofibrillar proteins (actin and myosin) gradually reach their pI (isoelectric point) at which point no more water molecules are able to bind to the proteins because they lose their charges. This will cause gaps between the myofibrils that create light scattering and increase reflectivity.

At the surface of the meat (top few millimeter), myoglobin binds oxygen and forms oxymyoglobin over a period of 10 or more hours. At this point, myoglobin still competes with the mitochondrial respiratory system and the rate of oxygen diffusion into the meat is less than the rate at which the mitochondria consume oxygen. The net result of oxygen diffusion is a decrease in oxygen concentration from the surface to the interior of the meat.

If the meat is stored for a period of time, the oxygen consumption rate will be reduced so that the myoglobin can bind oxygen more rapidly and as a result the oxymyoglobin layer becomes thicker. A typical type of chill-stored meat on display under oxygen-permeable wrap in a supermarket, its browning is first noticed in the area between the meat surface and the inner meat tissues where the oxygen partial pressure is low because at this point, metmyoglobin formation is maximized (1.5 mm). Over time, the browning migrates inward and outward until the surface of the meat is completely brown.

2.7. Colour stability and meat discolouration

2.7.1 Myoglobin and lipid oxidation

Lipid oxidation is a major deterioration reaction that often results in a significant loss of meat product quality. It has been established that lipid oxidation can cause meat discolouration due to the coupled reactions between lipid and pigment oxidation; however, the underlying mechanism for this is yet to be understood (Liu *et al*., 1995).

Spontaneous lipid radical formation or direct reaction of unsaturated fatty acids with molecular oxygen is thermodynamically unfavourable due to the spin restriction which prevents direct addition of triplet state oxygen to singlet state unsaturated fatty acid molecules. However, this can be overcome through photooxidation and partially reduced or activated oxygen species (hydrogen peroxide, superoxide anion, and hydroxyl radical). The rate of oxidation in meat products is affected by many extrinsic factors including temperature, meat processing operations, packaging of the meat and the

employment of non-meat ingredients in formulations. The rate of oxidation increases as the temperature rises (Gutteridge and Halliwell, 1995); hence, the storage of meat in a refrigerator or freezer can retard oxidative deterioration. In many cases, the exclusion, or reduction, of oxygen content via vacuum or modified atmosphere packaging will also minimize oxidation processes (Monahan, 2000). During meat processing, any physical manipulations that disturb the integrity of muscle tissue, such as mincing and flaking, can expose meat lipids to a pro-oxidative environment; this is why fresh meat has a lower oxidation rate than processed meat (Monahan, 2000).

Many researchers also believe that the presence of transition metals, notably iron, is pivotal in the generation of species capable of abstracting a proton from an unsaturated fatty acid (Gutteridge and Halliwell, 1990). Lipid hydroperoxides are fairly stable at physiological temperatures but in the presence of the transition metal complexes, their decomposition is greatly accelerated. Transition metals may initiate lipid oxidation by several mechanisms: 1) generation of unsaturated fatty acid radicals by single-electron transfer or hydrogen abstraction, 2) reaction with triplet oxygen to generate the superoxide radical, 3) indirect generation of oxygen species by oxidizing flavin cofactors, and 4) interaction with oxygen or peroxides (or iron containing enzymes and protein) to raise the metal oxidation state (Kanner *et al*., 1987). The rates of prooxidant activity of transition metals were discovered to occur in the following order: Fe^{2+}> Cu^{2+}>Co^{2+}>Mb (Tichivangana and Morrissey, 1985). The addition of antioxidants, or chelators, to meat often slows the rate of lipid oxidation (Igene *et al.* 1981).

2.7.2 Myoglobin oxidation leads to lipid oxidation

Many authors have shown that lipid and pigment oxidation reactions were closely coupled. This relationship has been proven by using liposome and microsome model systems (Chan *et al*., 1997). Gotoh and Shikima (1976) reported that the conversion of oxymyoglobin to metmyoglobin involved H_2O_2 and that oxymyoglobin oxidation was inhibited by the presence of catalase. In the presence of oxygen, oxymyoglobin is oxidized slowly to form metmyoglobin by releasing superoxide anion radicals ($O_2^{\bullet -}$) (Reaction 2.3). Superoxide dismutase catalyzes the superoxide anion radical to form water and hydrogen peroxide (H_2O_2) (Reaction 2.4). Later, Kanner and Harel (1985) suggested that the removal of H_2O_2 by catalase inhibited the formation of H_2O_2-metmyoglobin and reduced lipid oxidation in microsome systems which could form ferryl radical species (ferrylmyoglobin and perferrylmyoglobin) and initiate lipid oxidation. It has been reported that this H_2O_2-activated metmyoglobin caused rapid oxidation in poultry skeletal muscle microsomes (Kanner and Harel, 1985). Moreover, hydrogen peroxide (H_2O_2) could then be involved in Fenton reactions to form the hydroxyl radical ($OH^{\bullet}$) which also could initiate lipid oxidation (Reaction 2.5). In short, the oxidation of oxymyoglobin produces two prooxidant species that can enhance lipid oxidation: metmyoglobin and H_2O_2. As in a study by Rhee (1988), the catalytic activity of H_2O_2-metmyoglobin increased up to a molar ratio of 1: 0.25 metmyoglobin and H_2O_2, respectively. Rhee (1988) also concluded that H_2O_2-metmyoglobin was a better catalyst in lipid oxidation than the non-haem iron released from metmyoglobin by H_2O_2.

$$\text{Oxymyoglobin - } Fe^{2+} + O_2 \rightarrow \text{Metmyoglobin - } Fe^{3+} + O_2^{\bullet -} \qquad \text{(reaction 2.3)}$$

$$2O_2^{\bullet -} + 2H^+ \rightarrow O_2 + H_2O_2 \qquad \text{(reaction 2.4)}$$

$$Fe^{2+} \rightarrow Fe^{3+} + e^- \qquad \text{(reaction 2.5)}$$

$$H_2O_2 + e^- \rightarrow OH^{\bullet} + OH^-$$

$$\overline{H_2O_2 + Fe^{2+} \rightarrow OH^{\bullet} + OH^- + Fe^{3+}}$$

The inclusion of both SOD and catalase caused a lower metmyoglobin formation and TBARS values in control. However, the addition of SOD or catalase alone does not repress the formation of metmyoglobin and TBARS as much as if they are added together. Indeed, SOD has less ability to reduce metmyoglobin formation and TBARS than does catalase. Chan *et al*. (1997) concluded that the superoxide anion generated from oxymyoglobin oxidation would not be directly involved in lipid oxidation and H_2O_2 appeared to be an important mediator for oxymyoglobin and lipid oxidation. This implies that hydrogen peroxide (H_2O_2) is a better catalyst than superoxide anion radical ($O_2^{\bullet -}$) in lipid and myoglobin oxidation.

2.7.3 Lipid oxidation leads to myoglobin oxidation

Free radicals and aldehydes from the oxidation of muscle lipids and liposomes are water soluble so they can enter the cytoplasm to react with oxymyoglobin and accelerate metmyoglobin accumulation (Schaefer *et al*., 1995). Furthermore, they can also indirectly disturb the myoglobin-reducing systems (Faustman and Cassens, 1990a; Lee *et al.,* 2003).

Lipid oxidation can produce a wide range of secondary aldehyde products that are more stable than free radical species. The molecule 4- hydroxynonenal (4-HNE) is one of α, β-unsaturated aldehyde formed by oxidation of ω-6 unsaturated fatty acids (Lynch and Faustman, 2000). 4-HNE has the ability to covalently attach to myoglobin, and once attached, it accelerates myoglobin oxidation *in vitro* (Faustman *et al*., 1999).

Phillips et al. (2001) suggested that the 4-HNE may induce the alteration in the tertiary structure of oxymyoglobin and lead to conformational changes that make it susceptible to oxidation. This adduction occurs particularly in the histidine and lysine residues of myoglobin. Different species may have different myoglobin and lipid stability due to differences in amino acid sequence and fatty acid profile. For example, pork myoglobin has 9-histidine residues; on the other hand beef has 13-histidine residues in its myoglobin. This suggests that beef is more susceptible to myoglobin and lipid oxidation than pork. Furthermore, 4-HNE also has the ability to complex with metmyoglobin and it enhances the ability of metmyoglobin to catalyze lipid.

2.8 Effect of bacterial growth

The type and number of microorganisms present are important as they affect the rate of meat spoilage. Microorganisms that eventually reproduce to sufficient numbers to cause spoilage are those that find existing conditions most favourable. There are three types of microorganisms that are able to grow on the surface of meat: bacteria, yeasts, and moulds. Bacteria generally grow faster than yeasts, and yeasts outgrow moulds (Warris, 2000). *Pseudomonas* spp. are the most common and important of the spoilage bacteria found on both red meats and poultry under aerobic conditions. In the presence

of oxygen, meat has the symptoms of microbial spoilage, as described in Table 2.4. Under anaerobic conditions, however, normal aerobic flora is suppressed, and lactic acid-producing bacteria such as *Lactobacillus spp* are favoured (Hedrick *et al*., 1989).

Table 2.4: Typical symptoms of microbial spoilage in meat

Type of microorganisms	**Characteristic symptoms**
Bacteria	Slimy at the meat surface; discolouration; gas production; off-odours; and fat decomposition
Yeast	Yeast slime; discolouration; off-odours and tastes; and fat decomposition
Moulds	Surface stickiness and whiskers; discolouration; odours and taints; fat decomposition

Adapted from Lawrie, 1991

Suitable temperature, moisture availability, osmotic pressure, pH and oxidation-reduction potential are essential factors that determine the rate of microbial growth on the surface of meat and meat products. Overall, temperature is the most important factor governing microbial growth. The rate of microbial growth increases as the temperature rises to a microbe's optimal temperature, and then declines as the temperature continues to increase. Meat spoilage organisms can be categorized into three classes based upon their optimum temperature range. Microorganisms that have their optimum growth at temperatures lower than 20°C are called *psychrophiles*. Those that have growth optima at temperatures higher than 45°C are called *thermophiles*. And lastly, microorganisms with growth optima between psychrophiles and thermophiles are called *mesophiles* (Hedrick *et al.,* 1989). A few degrees change in temperature may favour the growth of

totally different organisms and result in different types of spoilage (Warris, 2000). In general, the growth of the spoilage bacteria is inhibited at lower temperature.

Moisture is important for microbial growth, but some microorganisms have the capability to remain dormant for a period of time under conditions of low moisture. The availability of water for microbial growth can be measured and expressed in terms of water activity (A_w). The A_w is defined as the ratio of the vapour pressure of the sample in question to that of pure water at the same temperature. The rate of bacterial growth slows as the A_w decreases. High solute concentrations in meat products, such as high sodium chloride levels, tend to inhibit bacterial growth. There are, however, some salt-tolerant microorganisms (halotolerant) which have the capability to grow successfully under these conditions (Lawrie, 1991). The system pH is also an important determinant of microbial growth. The final *post mortem* pH of meat is significant for meat resistance to spoilage. The pH of meat selectively influences the types of microorganisms that can grow on the surface. Microorganisms will grow better at neutral pH than at lower or higher pH (Hedrick *et al*., 1989).

The oxidizing and reducing power of meat can be determined by measuring the redox potential. Some microorganisms require high redox conditions while others require low ones in order to achieve optimal growth. Aerobic microorganisms are favoured by a high redox potential while anaerobic microorganisms prefer a low potential. Facultative microorganisms are able to grow under either low or high redox conditions. Microorganisms also have the capability to alter the redox state of the meat so that the activity of other microorganisms may be restricted (Hedrick *et al*., 1989).

However, the influence of redox potential only prolongs the initial lag phase and not the eventual growth rate (Barnes and Ingram, 1956).

Although not extensively evaluated, several studies have reported a relationship between bacterial growth and colour deterioration of the fresh meat (Cornforth, 1994). Aerobic bacterial growth can accelerate oxidation of meat pigments that lead to meat discolouration (Lawrie, 1991) by increasing the rate of myoglobin autoxidation under aerobic conditions (Renerree, 2000) through the elevation of oxygen consumption and the reduction of partial oxygen pressure to the level critical for myoglobin oxidation (Cheah and Ledward, 1997). The bacterial load, however, should be at least at the level of spoilage (10^7 CFU/cm^2) or the oxygen consumption by bacteria will not significantly affect pigment oxidation (Bevilacqua and Zaritzky, 1986).

Furthermore, microorganisms also have the ability to change the meat pH and produce amino acids and amines through proteolysis and glycolysis that further induce the myoglobin autoxidation (Hedrick *et al*., 1989). Different species of aerobic bacteria may have different abilities to affect meat colour and these abilities are also influenced by environmental condition such as temperature and pH. Thus, any factors that can enhance the microbial growth will also increase of the rate myoglobin oxidation rate (Sofos *et al*., 2000) which then leads to meat discolouration. Moreover, it is important to note that anaerobic bacteria do not generally cause meat discolouration (Kropf *et al*., 1986).

Pseudomonas spp. are the dominant psychrotrophs found in aerobically packaged meat (Ayres, 1960). Butler *et al*. (1953) inoculated beef steak surfaces with *Pseudomonas* and reported that the metmyoglobin formation rate was maximal during

the log phase of bacterial growth. Bacteria would reduce the oxygen partial pressure at the meat surface to the critical level for maximum metmyoglobin formation. However, Bevilacqua and Zaritzky (1986) argued that the oxygen demand by bacterial populations of 10^6 CFU/cm^2 was insufficient to facilitate increased pigment oxidation via reduced oxygen partial pressures (Robach and Costilow, 1961). These organisms are nutritively versatile, but display strong repression of other catabolic pathways when glucose is available as a substrate (Gill, 1986). At the point where glucose is depleted as a substrate, pseudomonads start to use amino acids that then produce putrid and odiferous byproducts.

Some other slow growing microorganisms include lactic acid bacteria, psychrotrophic enterobacteria and *Brochotrix thermosphacta*; these organisms may also represent a major fraction of the spoilage flora. If lactic acid bacteria are predominant initially, they could dominate the spoilage flora by inhibiting some of the competing microorganisms by the production of bacteriocins. In this circumstance, the lactic organisms will tend to dominate the spoilage process even when the product is displayed in air (Gill and Jones, 1994). If Brochotrix *thermosphacta* persists as a substantial fraction of the flora, the offensive byproduct which is produces throughout its growth, irrespective of the availability of glucose, will accumulate to produce odours that are usually described as being reminiscent of sweaty socks (Grau, 1983).

Delaying the bacterial spoilage of raw meat is highly correlated to the hygienic condition of the products. Therefore, it is obvious that the reduction of the initial number of microorganisms could extend the time required before they reach number sufficient to

cause spoilage. As growth is exponential, large decreases are required for incremental increases in the storage life.

2.9 Effect of meat pH

The effect of pH on meat colour stability is important from the standpoint of both ultimate pH on *post rigor* muscle, and the rate of pH decline in the *pre rigor*, *post mortem* condition. The ultimate pH of normal meat is approximately 5.4 to 5.8 and, in general, low pH values favor the oxidation of myoglobin. It has been reported that beef with an ultimate pH greater than 5.8 was more colour stable than similar meat with an ultimate pH of 5.6. Moreover, a low pH environment accelerates the protonation of bound oxygen and favours the release of superoxide anion (Livingston and Brown, 1982).

The drop of pH and the ultimate pH *post mortem* affects the outcome of meat colour. The rapid fall in muscle pH while the muscle is still warm causes the denaturation of myofibrillar protein, and as mentioned before, and this can lead to the increase of refractivity and the loss of water holding capacity of the muscles. This results in pale, soft and exudative (PSE) meat (Figure 2.16).

On the other hand, if pH does not drop *post mortem*, more water is kept between the myofibrillar proteins so that the extramyofibrillar fluid phase decreases. This makes the muscle arrangement more compact so that less incident light is reflected and more light is absorbed by the meat. Furthermore, at high pH, the mitochondrial respiratory system is relatively active and vigorously consumes molecular oxygen, so less oxygen is

available for oxymyoglobin formation. These events result in dark, firm and dry (DFD) meat.

The pH values for PSE, normal and DFD are 5.4, 5.5 and 6.1. The higher pH in DFD meat should result in more metmyoglobin being formed since it has higher oxygen consumption activity. However, DFD meat has the lowest percent metmyoglobin content in comparison to normal and PSE meat. This proves that ultimate pH is more important than the oxygen consumption rate in determining the relative colour stability (Zhu and Brewer, 1998).

2.10 Effect of vitamins

Vitamin E is fat soluble and has the ability to prevent lipid oxidation and automatically retards metmyoglobin formation in meat exposed to air; thus meat colour stability of the animals can be improved through vitamin E supplementation (Young *et al*., 2001). The concentration of vitamin E in normal animal feed (grain) is low (0.5 μg/g of lean meat) and the concentration of vitamin E supplementation can achieve approximately 5.0 μg/g of lean meat (West *et al*., 1997).

Vitamin E has the ability to scavenge lipid free radicals, slowing the metmyoglobin accumulation. Recent evidence suggests that α-tocopherol can delay the release of α, β-unsaturated aldehydes as prooxidative products of lipid oxidation from biomembranes so that the oxidation of pigments can be delayed. Moreover, α-tocopherol is also suggested to have the ability to regenerate cytochrome $\boldsymbol{b}_5$ so that the regeneration of metmyoglobin can be promoted.

Vitamin C possesses reducing power so it is able to reduce metmyoglobin and regenerate vitamin E. If an animal is orally supplemented with vitamin C, the concentration of plasma vitamin C increases sharply but it will fall quickly to homeostatically-controlled concentration. Therefore, vitamin C cannot be given to animals through feeding. It is more effective to intravenously inject vitamin C immediately before slaughter and in this way, the meat colour stability can be maintained *post mortem* (Hood, 1975). The combination of vitamin E and vitamin C supplementation has a greater effect in maintaining colour stability *post mortem* than individual vitamin supplementation.

2.11 Effect of storage condition in retail market

2.11.1 Temperature

The solubility of myofibrillar protein decreases as water between myosin and actin freezes so that they are arranged in more compact arrangement. This changes the optical properties so that the meat becomes less reflective and looks darker. Moreover, the formation of ice crystals also causes membrane damage so that it can enhance tissue degradation which then leads to the formation of free radical species (Young and West 2001). These free radical species again enhance the myoglobin oxidation to metmyoglobin.

Frozen meat may be thawed and cut before display. After thawing, myoglobin in the freshly cut meat surface binds oxygen and forms oxymyoglobin, but the meat will remains less reflective than meat that never was frozen so it appears darker. Over time,

the colour of frozen meat becomes dark red-brown due to a combination of low light reflection, surface drying and metmyoglobin formation. In addition, meat that has been previously frozen has a shorter display life on thawing (Moore, 1990).

Meat is usually sold in the chilled condition in the retail market. Meat starts to freeze at 1.5°C, and meat that is stored between this temperature and ambient temperature can be considered to be chilled (Young and West, 2001). In the retail market, the stability of meat colour is essentially influenced by the temperature of meat display because when the temperature increases, the rate of myoglobin oxidation is greatly accelerated due to the elevation of the rate of any prooxidant reactions within the tissue. The activity of the mitochondrial respiratory system increases as temperature increases oxygen consumption rate and reduces oxygen partial pressure to the point where metmyoglobin formation is maximized. Therefore, meat discolouration is delayed at low temperature storage because the oxygen consumption by tissue, lipid oxidation and microbial growth are inhibited.

2.11.2 Display condition

The display period is the duration that products are offered under lighting in the refrigerated retail display. The display period is not the same as the storage period because products are usually stored in the dark and usually not for sale when in storage. The perceived colour of meat in the retail display cabinets is affected by the lighting, either naturally through windows, or by incandescent or fluorescent light in display cabinets.

Colour temperature for meat display lighting ranges from 2600K, which is considered warm light with higher proportion of red wavelengths, to 4200K, which is a colder colour with higher proportion of blue wavelengths. Light that has red emission is good in bearing meat colour and it has been reported that lighting with 21.4% - 34.4% red has a good colour rendition. Incandescent light has lower proportion of blue wavelengths, whereas cool white, deluxe cool white and deluxe warm fluorescent light are higher in green wavelengths. Therefore, incandescent light has more red wavelengths in its spectrum which make meats look more desirable in the display. However, incandescent light could cause a rapid increase in metmyoglobin, resulting in more undesirable colour due to the elevation if the surface temperature of the products (Calkins *et al*., 1986). Therefore, most of the meat display lights are of the fluorescent rather than incandescent type because fluorescent lights produce less heat than do incandescent lights. Deluxe fluorescent lights radiate about one-fifth as much heat as incandescent lamps on an equal foot-candle intensity of lighting basis.

Display lighting effects on the appearance or rate of discoloration of meat could result from: temperature elevation at the meat surface, photochemical effects, and spectral energy distribution patterns. Radiant heat from intense display lighting increases the temperature on the meat surface. Temperature of the meat surface increases proportionally with light intensity. This higher temperature also induces oxidation and microbial metabolism so that it speeds up deteriorative events at the meat surface. It has been reported that a greater pigment oxidation occurs in meat stored under light versus dark conditions because the microbial growth is enhanced under illumination (Djenane *et al*., 2001).

Light also has the ability to promote metmyoglobin formation through photochemical autoxidation. Shorter wavelengths, particularly in the ultraviolet range, are the most damaging (Young and West, 2001). Seyfert *et al*. (2006) showed that pork patties stored in the dark for 7 days and then in the light for 3 days were less discoloured (decreasing a*, b* and chroma) than those stored in the light for 10 days. This effect by light is often called photooxidation, and refers to the modification or destruction of amino residue side chain groups, DNA, or lipids by singlet oxygen which is produced from the interaction of ground state triplet oxygen and a photo-energized sensitizer molecule such as haem protein (Korycka-Dahl and Richardson, 1980). Any modification of the globin protein structure by photooxidation could result in rapid pigment oxidation.

Display lighting intensity can have an important influence on product display life. Discoloration was closely related to foot candle intensity times the light exposure time. Barbut (2001) reported meat display lighting in five surveyed meat counters to range from about 60 to 100 foot candles. More intense lighting is likely used now, partly because of use of multi-shelf meat display cases with lights at all levels. Use of high intensity lighting may result in more, and faster, meat sales, but if sales do not keep pace with discoloration, loss of profit may result overall.

2.11.3 Packaging

The most common method for meat packaging is the polystyrene tray with the drip pad and oxygen-permeable film overwrap to protect the meat from contamination and drying. The property of the overwrap is essential because it regulates the gaseous

environment in the package. It is desirable to have a low water vapour transmission rate to minimize moisture loss and a high oxygen transmission rate to satisfy the need of oxygen in oxymyoglobin formation (Hedrick *et al*., 1989). The use of PVC overwrap can enhance the red colour perception because it has high oxygen permeability (8000-12000 $cc/m^2/day/atm$). PVC is the most common, and least expensive, overwrap used in retailing (Reneree, 2000).

Vacuum packaging is another alternative for packaging. Laminates which have low water vapour and oxygen transmission rates are usually used in this type of packaging. When the oxygen diffusion is stopped, most of the pigments are gradually transformed to the purple reduced form of myoglobin because the respiratory enzymes in muscles and microorganisms use the oxygen available within the package (Hedrick *et al*., 1989).Vacuum packaging inhibits myoglobin autoxidation and spoilage by aerobic bacteria, but the red purple colour of myoglobin is not as acceptable to the consumer. Therefore, this type of packaging is usually used only for bulk packaging.

In order to achieve a condition that preserves the red colour of meat, prevents anaerobic spoilage and inhibits the growth of aerobic microorganisms, efforts have been made to control the gaseous atmosphere within the packaging. Accordingly, modified atmosphere packaging with 20% CO_2 and 80% O_2 have been used for flushing packages prior to sealing. The growth aerobic microbes is inhibited by 20% CO_2 (Reneree and Labadie, 1993); whereas, the presence of 80% oxygen promotes the formation of oxymyoglobin that can give a desirable meat colour but may also enhance the rate of autoxidation. As a result, this type of packaging offers only a slightly longer shelf-life than normal oxygen permeable packaging (Hedrick *et al*., 1989).

2.12 Endogenous antioxidants

The ultimate lipid oxidation potential of a meat and meat products is dictated by the balance between prooxidants and antioxidants. The mechanisms preventing or delaying oxidation reactions through the inhibition of lipid oxidative catalysts, free radical formation from pre-existing peroxides and free radical scavengers are still subject to much debate (Chan and Decker, 1994). The production of hydrogen peroxide (H_2O_2) by either the oxidation of oxymyoglobin or through other routes could form a highly-reactive hydroxyl radical in the process. Therefore, it is clear that any factor or event that can decrease hydrogen peroxide production would also affect the rate of lipid oxidation in meat.

Endogenous antioxidant systems in meat have been reported to play important roles in inhibition of lipid oxidation, providing they remain stable during storage (Pradhan *et al*., 2000). Antioxidant defense systems in muscle tissue also continue to operate *post mortem,* but their effectiveness decrease over time (Monahan, 2000). Some of the endogenous systems are enzymatic in nature. Important endogenous antioxidant enzymes include superoxide dismutase (SOD), glutathione peroxidase (GSHPx) and catalase (CAT).

Catalase and GSHPx are considered the major peroxide removing enzymes that are located in the cytosol (Chan and Decker, 1994). The activity of endogenous enzymes however, differs between species and muscle type (Hernadez et *al*., 2002; Pradhan *et al*., 2000). Even animals in the same species can have different antioxidant activities and this variation may due to variations in genetic types so that oxidative stability between meats

is not the same (Hernandez *et al*., 2004). Rodriguez-Martinez and Ruiz-Torres (1992) indicated that the level of endogenous antioxidant enzymes increased during aging and this elevation could be interpreted as a positive feedback mechanism in response to rising lipid peroxidation.

2.12.1 Superoxide Dismutase (SOD)

SOD is an endogenously-produced enzyme essentially present in every cell of the body. Cellular SOD is actually represented by a "group" of metalloenzymes with various prosthetic groups. SOD appears in three forms: Cu-Zn SOD in the cytoplasm, Mn-SOD in the mitochondrion and Cu-SOD in the extracellular matrix. This enzyme catalyzes the superoxide anion radical ($O_2^{\bullet -}$) to form hydrogen peroxide (H_2O_2) (Reaction 2.4, Gutterridge and Halliwell, 1994).

2.12.2 Catalase (CAT)

Catalase is an enzyme that contains haem-bound iron at its active site. It is present in the tissues of all body organs and is especially concentrated in the liver and erythrocytes. It catalyzes the conversion of H_2O_2 to water and oxygen (reaction 2.6).

As previously mentioned, the reaction of $O_2^{\bullet -}$ is catalyzed by SOD to give H_2O_2. An increase in the production of SOD without a subsequent increase in the activities of CAT or GSHPx leads to accumulation of H_2O_2, which is eventually converted to the hydroxyl radical ($HO^{\bullet}$). It is therefore very important to balance the H_2O_2 generating and dismutating enzymes (Gutterridge and Halliwell, 1994).

2.12.3 Glutathione Peroxidase (GSHPx)

The glutathione system (*i.e.*, glutathione, glutathione peroxidase and glutathione reductase) is a key defense system that protects cells against the build up of H_2O_2 and other peroxides (Reaction 2.7). The glutathione redox cycle is a central mechanism for the reduction of intracellular hydroperoxides via the oxidation of glutathione (GSH).

$$H_2O_2 + 2GSH \rightarrow GSSG + 2H_2O \qquad \text{(Reaction 2.7)}$$

Reduction of the oxidized form of glutathione (GSSG) is then catalyzed by the enzyme, glutathione reductase, in the presence of the reduced form of the coenzyme, nicotinamide adenine dinucleotide phosphate (NADPH) (Reaction 2.8).

$$GSSG + NADPH + H^+ \rightarrow 2GSH + NADP^+ \qquad \text{(Reaction 2.8)}$$

It is important to note that glutathione peroxidase needs selenium for its activity. There are four forms of glutathione peroxidase (GSHPx) enzymes (Gutterridge and Halliwell, 1994): cystolic Glutathione Peroxidase (cGSHPx) which is ubiquitously distributed, Phospholipid Hydroperoxidase Glutathione Peroxidase (PHGSHPx) which is in plasma membranes to reduce hydroperoxides of complex lipids, plasma Glutathione Peroxidase (pGSHPx) which is in blood plasma and Gastro-Intestinal Glutathione Peroxidase (GIGSHPx) which is in the liver and GI tract. Both pGSHPX and PHGSHPx

can counteract LDL peroxidation in plasma and endothelial cells, whereas GIGSHPx protects against dietary hydroperoxides (Gutterridge and Halliwell, 1994).

2.13 Non-meat ingredients

The addition of non-meat ingredients to processed meats can be considered as food additives or food adjuncts. A food additive is any substance that becomes part of a food product, either directly or indirectly, during some phase of processing, packaging or storage. They can either be derived from naturally-occurring or synthetic materials. Direct additives are purposefully added to food to serve a specific function. Indirect additives become part of food in very small quantities as a result of growing, processing or packaging. Food additives tend to increase the availability, quality and safety of foods while keeping costs low, and can be grouped into the following categories:

- Preservatives, which help keep food fresh and prevent spoilage by controlling bacteria, mould, fungi, yeast, or chemical changes;
- Nutrients, which maintain or improve the nutritional quality of food;
- Processing aids, which make products more pleasing (often for sensory purposes) by improving their consistency, providing body, adding stability, helping oil and water mixtures, retaining moisture or preventing lumping;
- Flavours, which complement, magnify, or modify the taste and aroma of a food. These can include spices, flavour enhancers, natural and synthetic flavours and sweeteners; and
- Colours, which give foods a desired, appetizing or characteristic appearance.

In meat processing, there are normally two types of additives used in meat formulations: meat and non-meat additives. A meat additive is the blending of meat from two or more different species; whereas, non-meat ingredients are adjuncts that are included for specific purposes to obtain desired effects in a processed product. Some examples of important non-meat ingredients are described below.

2.13.1 Sodium chloride (salt)

Salt (NaCl) is very important for the overall texture of the meat products. Sodium chloride is the most common salt that is used as a non-meat ingredient. It helps solubilize and extract myofibrillar proteins (Xiong and Mikel, 2001). Besides having important sensory functions, salt also can have a preservative action. It can inhibit microbial growth by lowering water activity, but must be present at certain concentrations to achieve this effect (Hedrick *et al.*, 1989). It has also been reported that sodium chloride can have pro-oxidative activity in the concentration range of 0.5% to 2.5%, which is typical for meat products (Rhee, 1999). This prooxidant activity can accelerate lipid oxidation (Tan and Shelef, 2002) and also limit the activity of endogenous enzyme (Hernández *et al.*, 2002). Lee *et al*. (1997) concluded that NaCl compromised the antioxidant activity in muscle by decreasing the catalytic activity of endogenous antioxidant enzymes.

NaCl has the ability to disturb the structural integrity of membranes, thereby bringing catalysts into closer contact with lipid reactants. This effect is the same as the

effect of cooking on muscle membrane systems (Rhee, 1988). It has been reported that non haem iron content tended to be higher as NaCl concentration increased in beef and chicken. In addition, Kanner *et al*. (1991) also suggested that NaCl disturbs the interaction between iron ions with proteins so that more free iron ions could interact with lipid to induce lipid oxidation. Moreover, free radicals from the accelerated lipid oxidation might have further increased the oxidation of haem pigments. NaCl may also initiate lipid oxidation by promoting the formation of hypervalent ferrylmyoglobin or activated metmyoglobin.

2.13.2 Sodium erythorbate

Sodium erythorbate is a D-isomer of sodium ascorbate but only has antioxidant function and does not possess any significant vitamin activity (Deshpande *et al*., 1995). Besides being less expensive than ascorbate, erythorbate reduces constituents in meat quicker than ascorbate; hence, it is more commonly employed by meat processors (Madhavl and Salunkhe, 1995). In cured meats, sodium erythorbate has three main functions: 1) it helps reduce nitrite to nitric oxide by maintaining reducing conditions in meat, thus accelerating the nitrosylation reaction of myoglobin and the formation of the heat-stable pink of cooked cured meat (Jadhav *et al*., 1995), 2) it functions as an antioxidant and contributes to colour stability of the product by reducing its sensitivity to colour fading (*i.e.*, photooxidation) under conditions of retail display, and 3) it helps prevent the formation of carcinogenic *N*-nitrosamines in cured products, especially bacon, by acting as an *N*-nitrosamine blocking agent. In non-cured meat, such as fresh

sausages, sodium erythorbate serves as primary antioxidant to delay discolouration. As an antioxidant, sodium erythorbate helps improve oxidation stability similar to that of vitamin C.

2.13.3 Lemon juice

Lemon juice, which contains citric acid, is added to processed meats as an acidulant. There are regulations in different countries as to the permissibility of this additive to various meat products. Citric acid is approved as an acidulant, cure accelerator, dispersing agent, flavouring agent, sequestering agent and as a synergist for antioxidants in a number of food systems (Deshpande *et al*., 1995). Haymon *et al*. (1976) showed that lemon juice had the ability to protect beef loaf from rancidity development. Citric acid is not considered to be a true antioxidant but it has the ability to enhance natural antioxidant activity by metal ion deactivation in the aqueous phase and also to alter the lipid-aqueous interfacial tension (Swern, 1964; Benedict *et al*. 1975). However, in their study, citric acid showed a limited effect in decreasing lipid and haem oxidation.

2.13.4 Toasted wheat crumbs or texturized soy protein concentrate

In North American, toasted wheat crumbs and texturized soy protein are commonly added as extenders, binders or fillers to meat products. They help bind excess water and stabilize both fat and water. These non-meat ingredient additives are important because they can improve water-holding capacity, enhance texture and flavour, reduce shrinkage during cooking, and reduce formulation costs (Hedrick *et al*.,

1989). Isolated soy protein contains approximately 90% protein and it does not compromise the flavour of the meat products if concentrations are maintained at a reasonable level (Hedrick *et al*., 1989). Toasted wheat crumbs, which are less expensive than isolated soy protein, are high in starch and relatively low in protein content (Hedrick *et al*., 1989), so they can bind large amounts of water.

2.13.5 Seasonings

A seasoning is any ingredient that can improve or modify the flavour of meat products, and how much a processor can add to a formulation is generally not dictated by regulations. Spices and herbs are aromatic plants. They are commonly added to meat products in the form of natural spices or as spice extracts. The addition of seasonings to a meat formulation is to create distinctive flavours and to help develop novel products. In some cases an added benefit lies in the fact that polyphenolic constituents in these aromatic plants provide preservative or antimicrobial effects. Certain spices, herbs and extracts have antioxidant activities and thereby reduce the rate of oxidation (Hedrick *et al*., 1989).

2.13.6 Alkaline phosphates

Food-grade phosphates are often used to improve quality. They can increase the water-holding capacity of meat and meat products so that the tenderness and juiciness of processed products are improved. The addition of alkaline phosphates, such as tetrasodium pyrophosphate, sodium tripolyphosphate and various phosphate blends,

increases the ionic strength so that meat proteins (notably actomyosin) are better solubilized. Moreover, alkaline phosphates increase the pH of meat leading to better water holding capacity and sliceability of the end product. Phosphate also has the capability to sequester metal ions. By acting as a secondary antioxidant, they help retard the development of oxidation. Overall, the addition of phosphate improves meat texture and maintains colour stability of the finished products (Roberts *et al*., 1991).

2.13.7 Antioxidants

Most synthetic antioxidants are of the phenolic type. In North America, the commercially available and currently used ones in food products include butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG) and *tert*-butylhydroquinone (TBHQ). The presence of electron-donating moieties at the *ortho* and *para* positions of the aromatic ring can react with free radicals, such as the hydroxyl radical. By quenching these reactive species, the initiation of autoxidation can be inhibited. In the neutralizing process of a free radical, a phenoxy radical is formed from the original antioxidant but this radical is stabilized by the aromatic ring and bulky groups attached to it. Their use is limited to certain types of fresh and precooked sausage, dry sausages and dried meats (Kabara, 1991).

2.13.8 Water

Water plays a very important role in processed meat because water is needed for emulsion formation in emulsified products and curing brines in cured meat products. As

mentioned before, metals have the ability to enhance lipid oxidation; therefore, it is very important not to use hard water as part of the non-meat ingredients because the contamination of metal ions from hard water accelerates lipid oxidation and then lipid oxidation promotes myoglobin oxidation that can reduce the stability of meat colour, and finally it can reduce the shelf life of meat colour (Montana Meat Process Convention, 2001).

Water is also a very important non-meat ingredient for reduced-fat meat products. The addition of extra water (50%-80% of meat block) to replace fat is commonly employed to produce low fat emulsion sausages (Rogers, 2001). It is cheap, has no calories and increases the bulk of the meat batter. However, it does not necessarily replace the functionality properties of fat, such as mouth feel, spice intensity and purge (Rogers, 1991).

2.14 Redox potential of meat and meat products

The redox potential is the affinity of a substrate to acquire electrons. Each substrate has its own intrinsic redox potential; their affinity for electrons is greater when a more positive potential exists. A single potential measurement, however, is indeterminate so redox potential needs a common basis, which is usually the standard hydrogen electrode (SHE). These redox potentials are referred to as E_h-values. Substrates with a negative voltage have a reducing capacity toward the SHE. On the other hand, if the substrate has a positive voltage relative to the SHE it has an oxidizing capacity.

The redox potential of meat changes over time because of intrinsic factors (microbiological and enzymatic activity) and extrinsic factors (atmospheric oxygen). The value of the redox potential after processing can offer, in some cases, a description concerning the characteristics of the product, such as, the age of the product (storage time) and if the length of storage is known, the storage conditions and the microbiological activities can be deduced (Rödel and Scheuer, 1999a). Thus, redox potential values may be used to assess the process of decomposition of fresh meat during storage.

Meats and meat products are comprised of many substrates and each of these can influence redox potential. It is difficult to determine the effect of each of those substrates on the overall redox potential of meat and meat products. However, groups of meat and meat products, which vary in compositions (ingredients and additives) and methods of production, have typical redox potentials. For example, the redox potentials of fresh meat are usually in the negative range. The median value for fresh pork is ~ -200 mV whereas that for fresh beef is ~ -250 mV (Rödel and Scheuer, 1999a). The redox potentials for cooked sausages are in the range of 0 mV to 60 mV and the redox potentials. It is apparent that the redox potentials of meat products increase after processing, due to grinding, which increases the surface area and exposes the meat to atmospheric oxygen. Thus, the initial measurement is important for further studies on the improvement of quality in meats and meat products (Rödel and Scheuer, 1999b).

The redox potential of the meats and meat products can influence the type of microorganisms that grow on the meat products' surfaces (Barnes and Ingram, 1955; Rödel and Scheuer, 2000b). Barnes and Ingrams (1955) reported that *Clostridium*

perfringens only initiated growth in fresh meat after the E'_h dropped to below -36 mV. The authors also observed that the metabolism of microorganisms and their end products can influence the redox potential so that the values of redox potential change over time. Moreover, the redox potential can create "hurdles" along with other parameters (A_w and pH) to inhibit the growth of undesirable microorganisms (Rödel and Scheuer, 2000a).

The redox potentials of meats and meat products can be influenced by the addition of non-meat ingredients as the part of processing. For example, the addition of sodium erythorbate leads to a reduction in redox potential values (Rödel and Scheuer, 2000a). On the other hand, the addition of sodium nitrate increases the redox potential (Rödel and Scheuer, 2000a). This application gives users some control in modifying or defining the end products in order to prolong the shelf-life.

3.0 Materials and Methods

3.1 Study 1: Assessing the stability of fresh sausages over time at 4°C

3.1.1 Experimental Design

The main objective of this study was to determine the stability of fresh pork sausages in terms of their chemical, colour, and microbiological characteristics (Figure 3.1) during a typical retail display period. To mimic retail display conditions, fresh sausages were placed under fluorescent lights (F40 CW, General Electric, Mississauga, ON, 850-1100 lux) for 5 days at a constant temperature of 4°C. Before the fresh sausages were displayed, they were held after production for 7 days in the dark at -1°C, as this represents a typical period before distribution. Besides the fresh sausages, ground pork was used to assess how the meat itself behaves during the storage and display periods without any addition of ingredients.

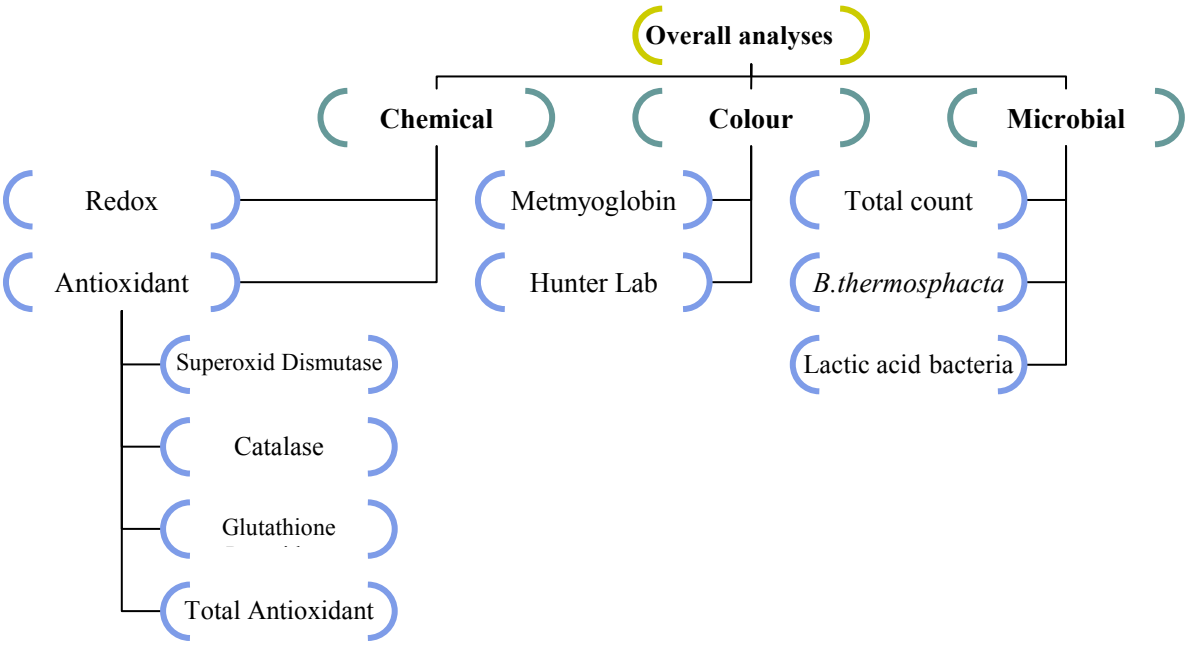


Figure 3.1: The experimental analyses performed on fresh sausages in patty form and ground pork conducted over days 1 to day 5 of simulated retail display at a constant 4°C and a light intensity of 850 – 1100 lux

Ten patties (P#1 to P#10) for each fresh pork and pork picnic sausage were produced on the production day (dp). On this day, all analyses, as depicted in Figure 3.1, were performed with the exception of the antioxidant analyses. Patty #1 (P#1) was followed from day 1 to day 5 of the display period for redox measurements. Additionally, duplicate patties (P#2 and P#3) were followed from day 1 to day 5 of the display period for colour measurements. Patties (P#4 to P#8) were sacrificed daily from day 1 to day 5 of the display period for antioxidant and microbiological analyses (Figure 3.2). This schedule was followed for both the fresh sausage patties and the ground pork patties. Three batches of sausages were prepared and analyzed to give true experimental replicates.

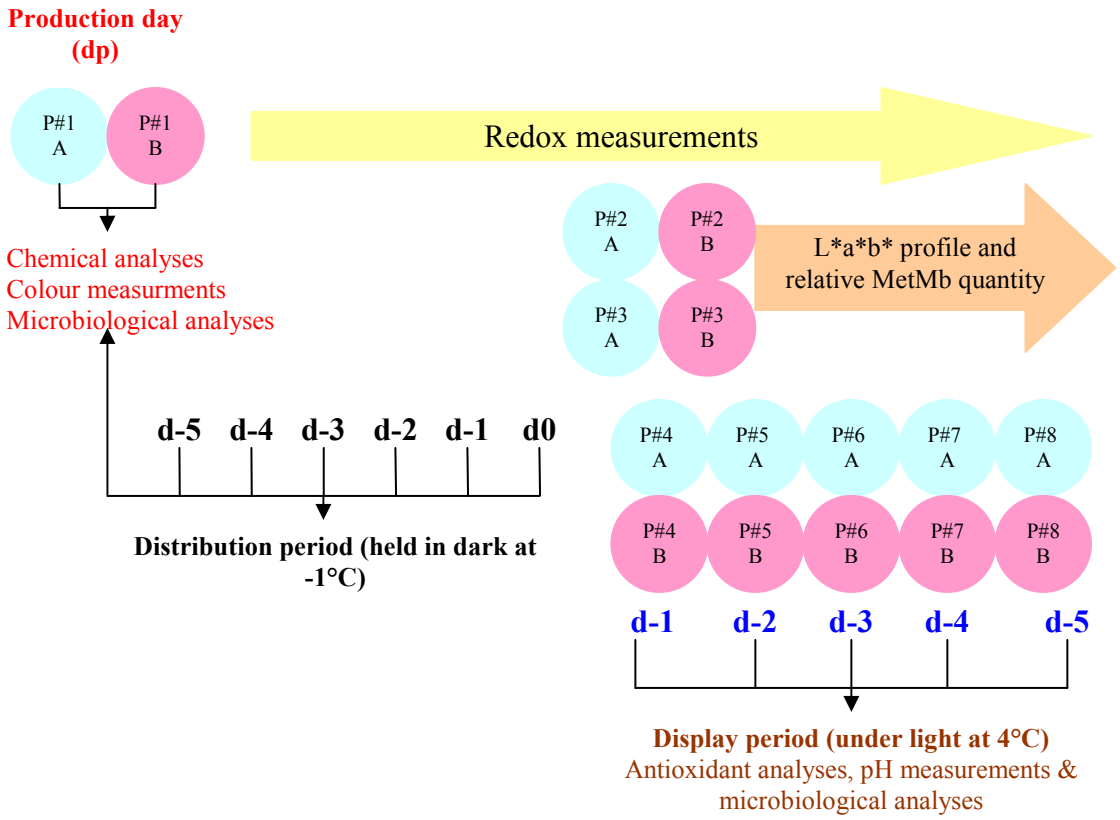


Figure 3.2: The sampling schedule: blue = fresh sausage patties; pink = ground pork patties

3.1.2 Meat processing

All meat processing was carried out in the Meat Pilot Plant located within the Department of Food and Bioproduct Sciences in the College of Agriculture & Bioresources at the University of Saskatchewan. A typical fresh sausage formulation was used in the preparation of pork patties; targets of 20% (or less) fat content and 14% to 16% protein content in the fresh sausages were specified. The non-meat ingredients included: water/ice (12%, w/w), salt (1.5%, w/w), lemon juice powder (0.25%, w/w), and sodium erythorbate 0.05% (*i.e.*, 500 ppm).

Pork picnic shoulder was obtained from Maple Leaf Foods and used for the formulation of fresh sausage within 10 days of the production date. The composition of the picnic shoulder boneless pork was estimated by using the Pork Picnic Shoulder - USDA Nutrition Database (Table 3.1). The pork picnic (handled in 6 kg batches) was first ground with a Hobart® grinder through an 11/16″ plate (The Hobart, Model 4812, Troy, OH, USA), after which random samples were taken for quick fat analysis (HFT 200, Data Support Co Inc., Encino, USA). A meat mixture with fat content ranging from 16 to 20 % was targeted before proceeding to the subsequent processing steps. After the required amount of fat content was achieved, the meat mixture was then ground twice using a 1/8″ plate. Some sample was taken at this point for proximate analyses (*i.e.*, crude fat, moisture and protein contents). A portion of the ground meat block (~ 3 kg) was then used in the formulation of fresh sausages with the specified non-meat ingredients: water/ice (12%, w/w), salt (1.5%, w/w), lemon juice powder (0.25%, w/w), and sodium erythorbate 0.05% (*i.e.*, 500 ppm). The remaining meat was formed into patties. All of the non-meat ingredients were first dissolved in the ice water before they were mixed together with comminuted meat to ensure that the non-meat ingredients were well-mixed. Both meat and non-meat ingredients were combined in a Berkel mixer (Model BA20, Omcan Inc., Mississauga, ON, Table 3.2) for 60 secs before they were portioned using the Omas patty stacker (Model BT10, Omcan Inc., Mississauga, ON) into ~120 g pork patties. All patties were then placed on Styrofoam® trays and over-wrapped with a standard oxygen permeable film (AEP Canada Inc., RMF61-HY, Scarborough, ON) with a known oxygen transmission rate of 1400 cm^3/0.06 m^2 per 24 hours. The patties were stored in the dark for 7 days at -1°C (*i.e.*, to simulate a typical

distribution scenario) before being placed under fluorescent lighting (850 to 1100 lux) at a constant 4°C for the remaining 5 days display period. Sixteen patties were made in total, with 8 patties prepared for each type (*i.e.*, either pork picnic or fresh pork sausage). Two calibrated thermometers were inserted into Erlenmeyer flasks filled with water held near the pork patties; the temperature was recorded daily over the entire storage period to confirm that a constant storage temperature was maintained.

Table 3.1: The composition of pork picnic shoulder based on the USDA Nutrition Database. Adapted from USDA Nutrition Data Laboratory, 2007

Proximate	**Units**	**Value per 100g**
Water	g	62.06
Energy	kcal	253.0
Energy	kJ	1059
Protein	g	16.69
Total lipid (fat)	g	20.19
Ash	g	0.840
Carbohydrate, by difference	g	0.000
Fibre, Total dietary	g	0.000

Table 3.2: The formulation for fresh sausage patties along with the calculated fat content and protein content

Ingredients	**Level (%)**	**Fat (%)**	**Protein (%)**	**Mass (g)**
Pork picnic shoulder	86.20	17.40	14.39	2586
Sodium chloride	1.500	0.000	0.000	45.00
Sodium erythorbate	0.050	0.000	0.000	1.500
Lemon juice powder	0.250	0.000	0.000	7.500
Water/ice	12.00	0.000	0.000	360.0
Total	100.0	17.40	14.39	3000

3.1.3 Colour measurements and haem pigment analyses

3.1.3.1 Instrumental colour measurements

Colour measurements (CIE, L^*, a^*, b*) were performed at the surface of the meat samples using a HunterLab colorimeter (MiniScan XE TM Version 3.0 1995) with Illuminant A and 10° standard observer for both pork picnic and fresh sausages. The colour measurement was taken with the film overwrapping left in place. Standardization of the unit was carried out with white and black tiles that were wrapped with the same over-wrap film as used for the patties (*i.e.*, standard oxygen permeable film, RMF61-HY). A pink tile, with specifications of $L^* = 76.39$, $a^* = 25.57$ and $b^* = 17.75$, was read to confirm that the unit was functioning properly before colour measurements of the meat patties were taken. Two patties for both pork picnic and fresh sausages were measured every day; 2 measurements (*i.e.*, at 90° rotation to one another) were recorded from each patty so that an average from 4 measurements could be reported.

3.1.3.2 Relative amount of metmyoglobin

The HunterLab MiniScab XE TM was also employed to determine the percent metmyoglobin at the surface of the meat patties. Accordingly, reflectance was measured in the form of K/S (Kubelka – Munk) values between 400 nm and 700 nm at 10 nm intervals. The relative content of metmyoglobin was estimated by calculating the ratio of $K/S_{572/525}$ as described by Hunt *et al*. (1991). K/S ratios at 572 nm and 525 nm, which were not given by the instrument, were calculated using linear interpolation. This

analysis was conducted to monitor changes of the relative amount of metmyoglobin on a day-to-day basis during storage, so that K/S conversion to absolute amount of metmyoglobin was not necessary. As the metmyoglobin content increases with storage, K/S ratios decrease correspondingly, so the K/S ratios were multiplied by -1 so that K/S ratios increase in the diagram (Lindahl *et al*., 2006a). Two patties for both pork picnic and fresh sausage were measured every day; two measurements (*i.e.*, at 90° rotation to one another) were recorded from each patty so that an average from 4 measurements could be reported.

3.1.4 Redox potential measurements

The measurement of meat patty redox potential was performed by using microelectrodes that were connected to a data logger (51x Micrologger, Campbell Scientific Inc., Edmonton, AB). The redox potential of fresh sausages and ground pork were measured over time. Two microelectrodes and 1 reference electrode were inserted into each patty with 1 microelectrode located near the patty's surface and other microelectrode was place into the middle portion of the patty (Figure 3.3).

Figure 3.3: Photograph showing meat patties with inserted microelectrodes wired to the data logger. Each patty had one microelectrode inserted near the surface of the patty, a second microelectrode inserted near the middle of the patty and a reference electrode in the middle of the patty

Redox potential values were determined using a AgCl microelectrode as a reference; the conversion of E to E_h (*i.e.*, standard hydrogen electrode as reference electrode, Equation 3.1) was not necessary because the trend of the redox potential values during both the first 7 days of storage at -1°C in the dark, and 5 days of storage at 4°C in the light, was more important than the exact values.

$$E_h = E + E_{ref} \qquad \text{(Equation 3.1)}$$

E_h = potential (mV) of sample solution relative to the standard hydrogen electrode.

E = potential (mV) of the sample measured at a specified temperature.

E_{ref} = reference electrode potential. For Ag : AgCl – saturated KCl a value of +199 ± 5 mV was used.

The efficacy of the microelectrode pair was checked in Zobell solution prior to the analyses in order to confirm that the microelectrodes were functioning properly. The Zobell solution consists of a 0.1 molal KCl solution containing equimolal amounts of $K_4Fe(CN)_6$ (potassium ferrocyanide) and $K_3Fe(CN)_6$ (potassium ferricyanide). The solution was prepared by dissolving 1.4080 g $K_4Fe(CN)_6 \cdot 3H_2O$, 1.0975g $K_3Fe(CN)_6$, and 7.4557 g KCl in deionized water and then diluting to 1 L. The E_h value of the Zobell solution is temperature-dependent (Table 3.3); thus, the E_h reading at room temperature should be 430 mV. The redox potential measurements were performed in the same cold room where the patties were stored, so that both light intensity and temperature could be held constant for all patties during the entire storage period.

Table 3.3: Eh of Zobell's solution as a function of temperature

Temperature °C	E_h (mV)
10	467
12	462
14	457
16	453
18	448
20	443
22	438
24	433
25	430
26	428
28	423
30	418
32	416
34	407
36	402
38	397
40	393

Adapted from Nordstrom and Wilde, 1998

3.1.5. pH determinations

Slurry pH measurements were conducted in duplicate every day from day 1 to day 5 for both pork picnic and fresh sausages. For each patty, 20 g of sample was blended with 80 mL distilled water and the pH values determined within 1 mins following blending. The pH meter (Fisher Scientific, Accumet AB15 plus Brinkmann Instrument Canada, Nepean, ON) was calibrated with pH 4.0 and pH 7.0 buffer solutions before the pH measurements were made. The measurements were performed in duplicate.

3.1.6. Extraction of meat endogenous enzymes

A 5 g sample of meat was aseptically taken from the surface of each designated patty and transferred to a 50 mL polypropylene Falcon® centrifuge tube. The tube was immersed in a beaker filled with ice water, and its contents were homogenized with 20 mL of 50 mM potassium phosphate buffer (pH 7.4) using a Polytron homogenizer (PT10135, Brinkmann Instrument Canada, Mississauga, Ontario) for 20 secs at 9000 rpm. The sample was then centrifuged (Beckman, model J2HC, rotor JA17, Beckman Coulter, Mississauga, ON) at 9000 rpm for 20 mins at 4°C. The resultant supernatant was filtered through filter paper (Whatman no 4, 25 μm particle retention, VWR Canlab, Mississauga, ON) into a graduated cylinder so that the volume of the extract could be recorded before its transfer to a 25 mL Erlenmeyer flask. This extract was used as a working solution for all antioxidant analyses. The volume of the working solution after extraction was measured for final calculation of enzyme activity.

3.1.7 Total antioxidant determination

The Trolox Equivalent Antioxidant Capacity (TEAC) procedure was used to measure the total antioxidant activity of meat extract, as described by Re *et al*. (1999). This method is based on the relative capacity of hydrogen-donating antioxidants to scavenge the pre-formed radical monocation of 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) [$ABTS^{\bullet+}$] compared to the antioxidant potency of 2.0 mM Trolox as a standard. One unit activity of TEAC is defined as the concentration (mmol/L) of Trolox having the equivalent antioxidant capacity of a 1.0 mmol/L solution of the substance under investigation (Miller *et al*., 1993). All the chemicals used in this analysis were obtained from Sigma Aldrich (Oakville, Ontario).

3.1.7.1 $ABTS^{\bullet+}$ preparation

The oxidation of 7 mM ABTS to $ABTS^{\bullet+}$ with 2.45 mM potassium persulfate was carried out by mixing 385 mg ABTS and 66 mg $K_2S_2O_8$ with 100 mL of 5 mM phosphate buffered saline (PBS, pH 7.4) in Erlenmeyer flasks. After 12-16 hours incubation, the $ABTS^{\bullet+}$ stock solution was filtered using filter paper (Whatman no 4, 25 μm particle retention, VWR Canlab, Mississauga, ON). This stock solution was then diluted with 5 mM PBS (pH 7.4) until an absorbance reading of 0.70 (± 0.02) at 734 nm was obtained. Note that the final absorbance reading of 0.70 (± 0.02) at 734 nm was made after the diluted $ABTS^{\bullet+}$ solution had been equilibrated at 30°C. The spectrophotometer (Agilent 8453 UV Visible, Agilent Technology, Mississauga, ON) was first zeroed with 5 mM PBS (pH 7.4) before sample readings were conducted.

3.1.7.2 Trolox standards

A 2 mM Trolox solution was prepared by weighing 50 mg 6-hydroxy-2, 5, 7, 8-tetramethylchromane-2-carboxylic acid into 100 mL of 5 mM PBS (pH 7.4). Serial dilutions were then performed to obtain Trolox working solutions with final concentrations of 2, 8, 12, 16, and 20 μM. The Trolox standard then was used to determine the activity unit of TEAC of the sample (Figure 3.4).

3.1.7.3 Spectrophotometric assay

After the addition of 10 μL Trolox standard or meat sample extract (1:2) to 1 mL of the $ABTS^{\bullet+}$ solution, the absorbance at 734 nm was measured using a spectrophotometer at 30°C at exactly 6 mins after initial mixing. The percent inhibition was then calculated as shown in Equation 3.2. A standard curve depicting % inhibition *vs.* concentration was constructed. The absorbances of the standards were read in duplicate for each concentration. The absorbances of the pork sample extracts were similarly measured in triplicate for both pork picnic and fresh sausages.

$$\% \text{ Inhibition} = \frac{Abs_{734} \text{ (blank – standard or sample) at 6 min}}{Abs_{734} \text{ (blank) at 6 min}} \times 100\% \quad \text{(Equation 3.2)}$$

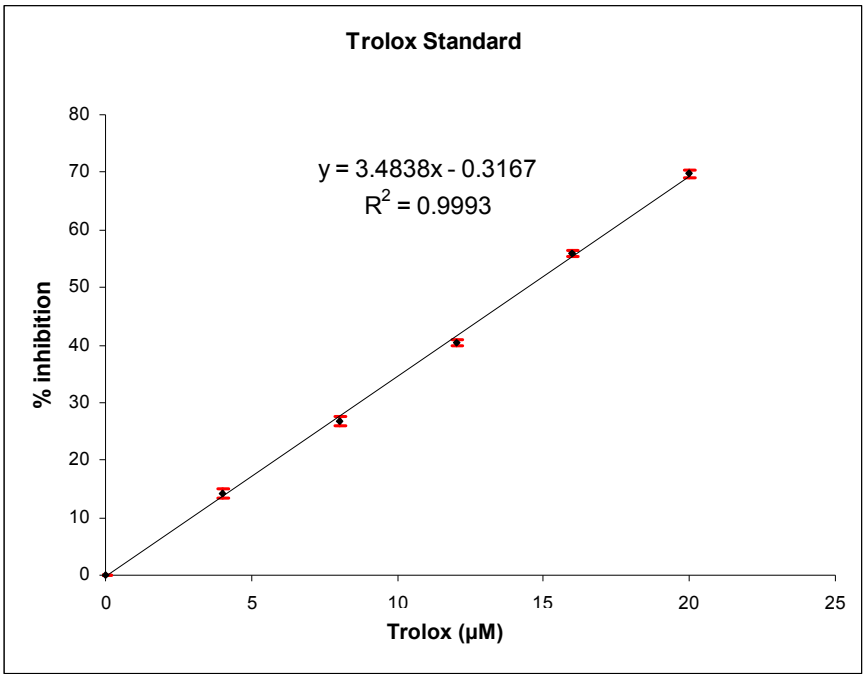


Figure 3.4: Trolox standard curve for the determination of TEAC value

3.1.8 Superoxide dismutase (SOD) activity measurements

The activity of SOD was measured using the method of Markulund and Markulund (1974). This method involves the inhibition of pyrogallol autooxidation in basic medium. The rate of pyrogallol autooxidation in the sample extract was then compared with the blank (*i.e.*, the phosphate extraction buffer) by measuring the increase in absorbance at 340 nm during the first 2 mins (in duplicate) at room temperature (Gatellier *et al*., 2004). The cuvette system was comprised of 50 µL meat extract or blank, 50 µL 10 mM pyrogallol and 1.9 mL 50 mM TRIS-Cacodylic Acid (+1mM DETPA) buffer One unit of SOD is defined as the activity of sample that inhibits the

reaction by 50% (Renerre *et al.*, 1996; Gatellier *et al.*, 2004). All the chemicals used in this analysis were obtained from Sigma Aldrich.

In this study, the sample standard was constructed by measuring the sample extract at different volumes (Table 3.4) so that percent inhibition (Equation 3.3) at different sample extract concentration can be obtained. From this curve (volume of sample extracts vs. percent inhibition), a straight line equation can be derived. From this equation, the volume of the meat extract (x value) that has 50% inhibition can be determined as one unit.

The undiluted meat extract must have a percent inhibition of greater than 50% in the cuvette system; this was achieved by adjusting the volume of meat extract and buffer in the cuvette system. In the case where a 50 μL meat extract sample with 0 μL added buffer demonstrated a % inhibition less than 50%, then the quantity of meat extract was increased to either 60 or 70 μL. In the sample standard, 5 points, including 0% inhibition in 0 μL meat extract sample with 50 μL buffer, were used to obtain the curve. The final SOD activity was expressed as U/ g of meat. The spectrophotometer was zeroed with 50 mM Tris-HCl buffer (pH 8.2) prior to reading the samples.

$$\% \text{ inhibition} = \frac{(\text{slope of blank} - \text{slope of meat extract})}{\text{slope of blank}} \times 100\% \qquad \text{(equation 3.3)}$$

Table 3.4: Meat extract dilution to establish sample standard

Meat extract (μL)	50 mM phosphate buffer pH 7.4 (μL)
50	0
40	10
30	20
20	30
10	40

3.1.9 Catalase (CAT) activity measurements

Catalase activity was determined by the method of Aebi (1983). Briefly, 1.0 mL of meat extract (diluted 1:8 with 50 mM phosphate buffer pH 7.4), 1.5 mL 50 mM phosphate buffer (pH 7.4) and 0.5 mL 100 mM H_2O_2 were added to a quartz cuvette. The activity of catalase in the meat extract was then determined in duplicate by monitoring the decrease in absorbance at 240 nm at room temperature during the first 30 secs for 1 sec interval. One unit of catalase activity is defined as the quantity of meat extract needed to decompose 1 μmol of H_2O_2/min (Hernández *et al*., 2002). The extinction coefficient of H_2O_2 (43.6/M cm) was used for the calculation of catalase activity (U/g of meat). The spectrophotometer was first zeroed with 2.0 mL 50 mM phosphate buffer (pH 7.4) and 1.0 mL meat extract (diluted with 50 mM phosphate buffer pH 7.4) prior to reading because the contribution of the haem meat pigments needed to be subtracted from the overall absorbance reading before the measurement of the catalase activity. All the chemicals used in this analysis were obtained from Sigma Aldrich.

3.1.10 Glutathione peroxidase (GSHPx) activity measurements

The enzymatic activity of GSHPx was measured using the indirect, coupled test procedure as described by Agergarrd and Thode Jansen (1982). The glutathione redox cycle is a central mechanism for the reduction of intracellular hydroperoxides by reducing H_2O_2 to H_2O via the oxidation of glutathione (GSH). The oxidized glutathione (GSSG) is reduced back to GSH by the enzyme GSH reductase (GR), a reaction requiring NADPH regenerated by glucose 6-phosphate dehydrogenase (G6PDH) (Gutteridge and Halliwell, 1989). All the chemicals used in this analysis were obtained from Sigma Aldrich.

GSHPx activity was measured with GSH reduction coupled to NADPH oxidation by the enzyme gluthathione reductase. The sample extract was added to the reaction medium (Table 3.5) and the rate of NADPH oxidation at 37°C measured at 340 nm in duplicate over 5 mins. This reading was taken 1 mins after the reaction was initiated via the addition of tert-butyl hydroperoxide, TBHP. GSHPx activity is the amount of extract that is required to oxidize 1 μmole NADPH/min at 37°C (Hernandez et al., 2004). The extinction coefficient of NADPH is 6330/M cm. The spectrophotometer was zeroed with a 16 mM EDTA in 400 mM phosphate buffer (pH 7.4) prior to sample measurements.

Table 3.5: The reaction medium in the cuvette for GSHPx determinations

	Volume (μL)
16 mM EDTA in 400 mM phosphate buffer (pH 7.4)	500
Glutathione reductase 15 U/mL	50
60 mM Glutathione reduced (GSH)	200
50 mM phosphate buffer (pH 7.4) or meat extract	100
Deionized water	400
Incubation for 10 min at 37°C	
3 mM NADPH in 0.1% (w/w) $NaHCO_3$	200
6.3 mM *tert*-butyl hydroperoxide (TBHP)	500

3.1.11 Microbial analyses

Ten gram samples of pork patties that had been aseptically scraped from the surface of the patties were homogenized in a Stomacher blender (Stomacher® LabBlender 400, St. Edmunds) with Seward BA6041/STR filter bag (VWR, Edmonton, AB) in 90 mL of sterile 0.1% (w/v) peptone water for 1 mins. The homogenate was serially diluted using 0.1% sterile peptone water before plating. Three plates were prepared for each dilution by spreading 0.1 mL of each dilution on agar plates. Three types of media were used to assess the microbiological flora present on both fresh sausages and ground pork:

- Trypticase Soy Agar (TSA;BBL, Becton Dickinson, Cockeysville, MD) + 0.1% (w/v) yeast extract (Difco Laboratories, Detroit, MI), incubated for 24 hours at

37°C for the isolation and cultivation of a wide variety of heterotrophic microorganisms;

- De Man Ragosa and Sharpe Agar (MRS; EMD Chemicals, Darmstadt, Germany) for 72 hours at 30°C for the cultivation of lactic acid bacteria; and
- Streptomycin Thallous Acetate Actidione (STAA; Oxoid, Nepean, ON) Agar incubated for 48 hours at 25°C aerobically for the cultivation of *Brochothrix thermosphacta* (Gardner, 1985).

Microbial enumeration was performed using the plate count method (spread plate) in triplicate and was presented as the number of colony forming units (CFU) per gram of meat.

3.1.12 Statistical analyses

The colour, chemical, and microbiological data were analysed as repeated measures with a Completely Randomized Design using the Mixed Model procedure of SAS (SAS Institute Inc., 1999). The mixed procedure was applied when calculating the least squares means (LSM) and standard error (SEM). Both treatments (*i.e.,* fresh sausages and ground pork) were analyzed in triplicate (3 batches) from day 1 to day 5, for each study. The Satterthwaite's approximation on degrees of freedom was used with the Kenward-Roger adjustment on standard errors. The pdiff test was used to determine the least significance difference between LSM. Significance was declared at $p<0.05$, or as otherwise indicated. Correlation coefficients among colour, chemical, and

microbiological analyses data were also performed using the Pearson's correlation of SAS (SAS Institute Inc., 1999).

3.2 Study 2 - Assessing the effect of non-meat ingredients on the stability of fresh sausages over time at 4°C

3.2.1 Experimental Design

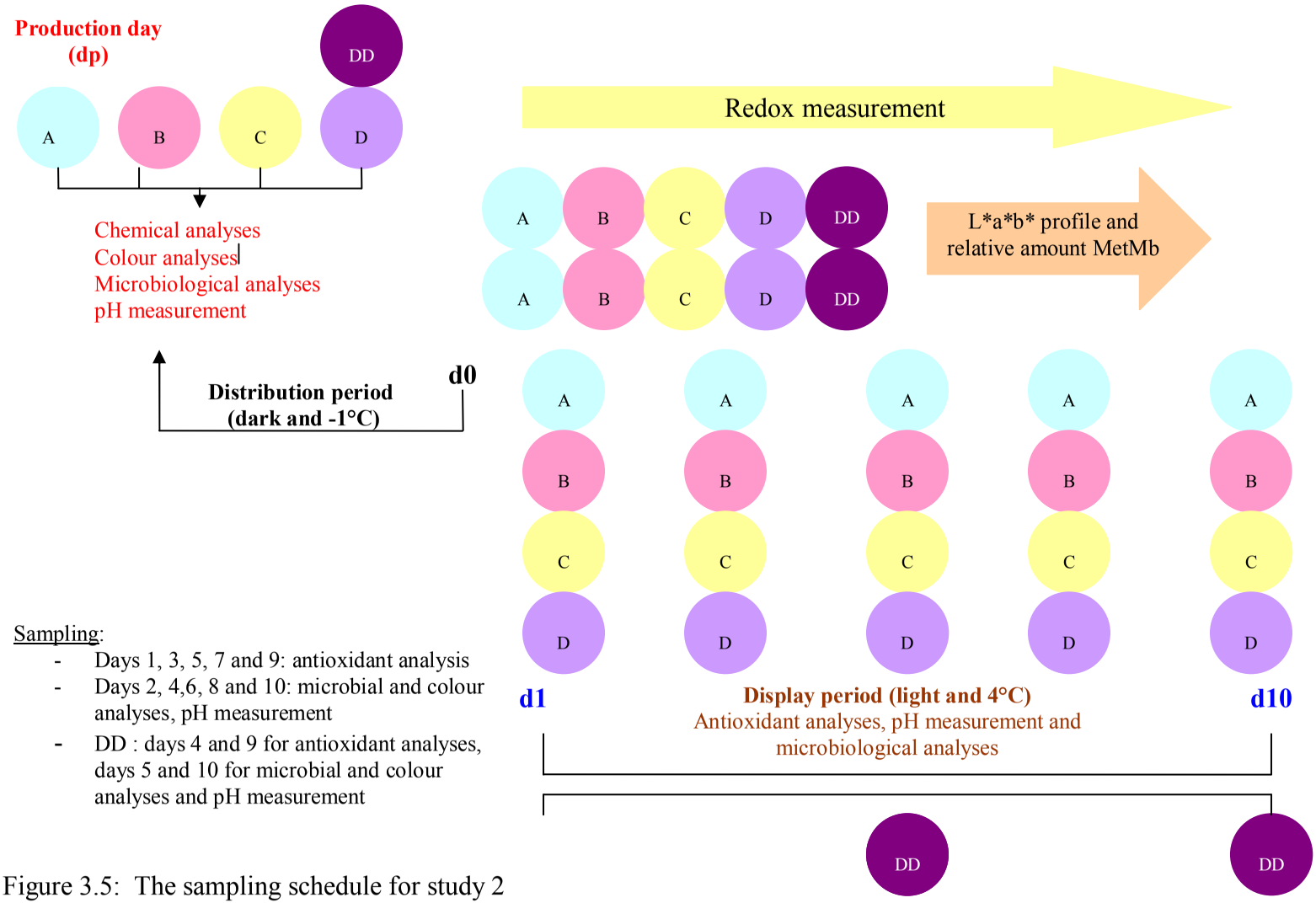
The objective of this study was to assess the effect of non-meat ingredients, which include lemon juice powder and sodium erythorbate, on the stability of fresh sausages in terms of their chemical, colour, and microbiological characteristics over time at a constant temperature of 4°C under fluorescent lighting of 850 -1100 lux. The lemon juice powder was obtained from Newly Weds Foods Co. (Mississauga, ON). It was comprised of lemon juice solids and maltodextrin, and had moisture content less than 5.5%. Nutritional facts of the lemon juice powder from Newly Weds Food Co (Mississauga, ON) are provided in Table 3.6. The citric acid content in lemon juice powder was measured by an acid titration method as described in section 3.2.3.

The sampling schedule is described in Figure 3.5. On the production day, 8 fresh sausages were made for each type of fresh sausage A, B and C. Twelve fresh pork "D" sausages were made as 4 extra fresh sausages were needed for the light analyses. During the display period, fresh sausages were displayed under the fluorescent light (General Electric F40 CW, 850-1100 lux) at constant 4°C for 10 days. Before the fresh sausages were displayed, they were held after production for 7 days in the dark at -1°C, representing a typical distribution time. The antioxidant analyses were performed on odd

days (day 1, 3, 5, 7 and 9). Two thermometers were inserted into water in the Erlenmeyer flasks near the pork patties and the temperature was recorded daily over the whole storage period to confirm that temperature was held constant during storage. The microbial analyses, colour analyses and pH measurement were performed on even days (day 2, 4, 6, 8 and 10). The effect of light was assessed by displaying 4 fresh sausages with no light at a constant 4°C for ten days. Two patties were kept for 10 days for colour analyses which were performed on days 6 and 10. Microbial analyses and pH measurements were also performed on days 6 and 10. Antioxidant analyses were performed on days 5 and 9.

Table 3.6: Nutrition facts per 100 g of lemon juice powder. Powder was obtained from Newly Weds Food Co

Nutrition	Value
Energy	397calories
Fat	0.00g
Saturated	0.00g
Trans	0.00 g
Cholesterol	0.00 g
Sodium	107 mg
Carbohydrate	91.2 g
Fiber	0.00 g
Sugars	5.00 g
Vitamin A	0.00g
Vitamin C	0.00 g
Calcium	1.00 mg
Iron	0.00 g



Sampling:

- Days 1, 3, 5, 7 and 9: antioxidant analysis
- Days 2, 4,6, 8 and 10: microbial and colour analyses, pH measurement
- DD : days 4 and 9 for antioxidant analyses, days 5 and 10 for microbial and colour analyses and pH measurement

Figure 3.5: The sampling schedule for study 2

In order to determine the effect of lemon juice powder and sodium erythorbate on the chemical, colour, and microbiological stability of 4 types of fresh sausage (Table 3.7), the proportion of all meat and non-meat ingredients were maintained as given in Study# 1; these ingredients were kept constant in each type of the fresh pork sausage. The meat ingredients, water/ice and salt were the same in all sausages, with lemon juice powder and sodium erythorbate being the variable ingredients.

Table 3.7: Type of sausages examined during this study

		Sodium erythorbate	
		0%	0.05%
		Type A	Type B
Lemon juice powder	0%	(0%, 0%)	(0%, 0.05%)
		Type C	Type D
	0.25%	(0.25%, 0%)	(0.25%, 0.05%)

All chemical, colour, and microbiological analyses (refer to Section 3.1.3 through Section 3.1.10) were performed as previously described with the exception of total antioxidant analysis (TEAC). Study# 1 revealed that the TEAC analysis did not give very much useful information regarding the antioxidant status of the samples unless other total antioxidant analysis, such as FRAP analysis, was performed along with TEAC analysis. Each type of fresh sausage was sampled as described previously (see Figure 3.1). Replication was also derived from 3 different batches of pork picnic meat for fresh pork production.

3.2.2 Meat processing

The production of fresh sausages was performed according to the same protocol as in Section 3.1.2 with the exception of the mixing step. Smaller portions of meat and non-meat ingredients, as indicated in Table 3.8, were blended in the mixer (Kitchenaid ®, model K45, Hobart MTG. Co.,Troy, ON) for 20 s at the lowest speed prior to formation of fresh sausages Type A, B, C, and D in the form of patties.

3.2.3 Citric acid determination

The content of citric acid in the lemon juice powder was determined by a simple acid titration. It is important to note that citric acid ($H_3C_6H_5O_7$) contains 3 carboxylic acid functional groups (Figure 3.6). When dissolved in water, the molecule exists as a triprotic acid capable of donating 3 protons. Each proton (H^+) from the carboxylic acid group will yield a molecule of water when citric acid reacts with strong base ($^-$OH).

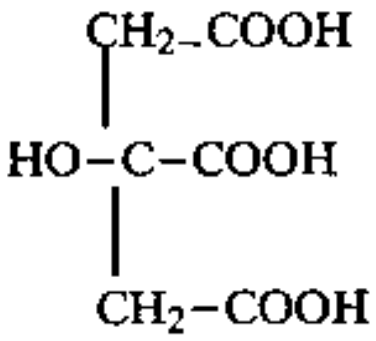


Figure 3.6: Citric acid as a triprotic acid

A 2% (w/v) lemon juice powder solution was prepared by weighing 2 g of lemon juice powder into a 100 mL volumetric flask and then filling it with deionized water. The pH was measured before titration with a standardized 0.1 N NaOH solution. Five mL of the lemon juice solution was quantitatively pipetted into a 250 mL Erlenmeyer flask. Then, ~30 mL deionized water and 3 drops of a 0.1% (w/w) phenolphthalein indicator prepared in 95% (v/v) ethanol were added to the flask. The volume of NaOH required to neutralize the lemon juice solution was recorded. The number of moles of citric acid in the lemon juice solution was calculated and the % (w/w) citric acid determined.

3.2.4 Statistical analysis

The results for colour, chemical, and microbiological data were analyzed as repeated measures with a factorial treatment design in a completely randomized design using the Mixed Model procedure of SAS (SAS Institute Inc., 1999). The 2 x 2 factorial treatment design was used with 2 levels of sodium erythorbate (0% and 0.05%) as one factor and 2 levels of lemon juice powder (0% and 0.25%) as the other factor. The mixed procedure was applied when calculating the least squares means (LSM) and standard error (SEM). All different 4 type of fresh sausages were analyzed in 3 batches f`rom day 1 to day 7. The Satterthwaite's approximation on degrees of freedom was used with the Kenward-Roger adjustment on standard errors. The pdiff test was used for least significance difference between LSM. Significance was declared at $p<0.05$, or as otherwise indicated.

Table 3.8: The formulation for Type A, Type B, Type C, and Type D with the calculated fat content and protein content

Ingredients	Level (%)	Fat (%)	Protein (%)	Weight (g)
Type A				
Pork picnic shoulder	86.20	17.40	14.39	1293
Salt	1.500	0.000	0.000	22.50
Sodium erythorbate	0.000	0.000	0.000	0.000
Lemon juice powder	0.000	0.000	0.000	0.000
water	12.00	0.000	0.000	180.0
Total	**99.70**	**17.40**	**14.39**	**1495**
Type B				
Pork picnic shoulder	86.20	17.40	14.39	1293
Salt	1.500	0.000	0.000	22.50
Sodium erythorbate	0.050	0.000	0.000	0.750
Lemon juice powder	0.000	0.000	0.000	0.000
water	12.00	0.000	0.000	180.0
Total	**99.95**	**17.40**	**14.39**	**1499**
Type C				
Pork picnic shoulder	86.20	17.40	14.39	1293
Salt	1.500	0.000	0.000	22.50
Sodium erythorbate	0.000	0.000	0.000	0.000
Lemon juice powder	0.250	0.000	0.000	3.750
water	12.00	0.000	0.000	180.0
Total	**99.75**	**17.40**	**14.39**	**1496**
Type D				
Pork picnic shoulder	86.20	17.40	14.39	1293
Salt	1.500	0.000	0.000	22.50
Sodium erythorbate	0.050	0.000	0.000	0.750
Lemon juice powder	0.250	0.000	0.000	3.750
water	12.00	0.000	0.000	180.0
Total	**100.0**	**17.40**	**14.39**	**1500**

4.0 Results and discussion

4.1 Study 1: Assessing the stability of fresh sausages over time at 4°C

4.1.1 Antioxidant status of meat patties

4.1.1.1 The effect of salt on endogeneous antioxidant activities

Overall, the catalase, SOD, GSHPx and total antioxidant activities were significantly higher in ground pork than in fresh sausages patties (Table 4.1 and 4.2). Salt may be responsible for this effect because salt has a prooxidant effect. This prooxidant activity can accelerate lipid oxidation (Tan and Shelef, 2002) and also limit the endogenous enzyme activities (Hernández *et al.*, 2002). Indeed, Lee *et al*. (1997) concluded that it compromised the antioxidant activity in muscle by decreasing the catalytic activity of endogenous antioxidant enzymes.

It is known that the oxidative stability of muscle foods can be altered by food processing operations and food additives. NaCl is known to have prooxidant traits over the concentration range of 0.5% - 2.0% (Rhee *et al.*, 1983) by compromising the ability of endogenous antioxidant enzymes since, in general, enzyme activities are affected by electrolytes and ionic strength (Richardson and Hyslop, 1985). NaCl may alter the reactivity of iron by the sodium ion activation of the chloride ion via myeloperoxidase (Kanners *et al.*, 1991; Kanner and Kinsella, 1983), and salt-induced changes in cellular organization (Shommer *et al.*, 1987).

4.1.1.2 Catalase

The catalase activity had an overall decreasing trend as seen in Table 4.3; however, the drop in catalase activity was more vigorous over day 1 to day 3 than from day 3 to day 5 for both ground pork and fresh sausages. This suggests that the depletion rate of catalase activity was higher at the beginning than at the end of the storage period. Lee *et al*. (1997) showed that catalase activities in ground pork boston butts (< 3 days *post mortem*) which was stored at -15°C decreased during the first week of storage, but became more stable thereafter. Renerre *et al.* (1996) also indicated that the catalase activity in beef that was stored 2°C wrapped in oxygen permeable film became more stable after 8 days of storage at 2°C. On the other hand, Pradhan *et al.* (2000) showed that the activity of catalase did not change notably in ground pork boston butt lean over 4 days of storage at 4°C, with an activity of 1144 U/g on day 0, 1092 U/g on day 2 and 1048 U/g for day 4. These values are much higher than the catalase activity seen in either ground pork and fresh sausages examined this study (Table 4.3). Biological variation or different type of meat could also cause these observed differences.

Pradhan *et al*. (2000) reported that antioxidative processes during storage and distribution of raw meat products is due to catalase activity, so its activity could potentially delay the oxidative process in stored meats. If catalase inhibits oxidation processes, it could in turn protect meat colour from discolouration since lipid oxidation can lead to meat dicolouration. The redness of the patty is the most important criteria for the consumer at the point of purchasing, and this redness is associated with a^* values. Catalase activity had significant positive correlation with a* in both ground pork (0.66)

and fresh sausages (0.80) patties (Table 4.4 and 4.5). This suggests that catalase could have a correlation in preserving meat colour in this study.

4.1.1.3 SOD

In this study, SOD decreased more rapidly from day 3 to day 5 than from day 1 to day 3; this trend was more obvious in fresh sausages than in ground pork (Table 4.1). In other words, the activity of SOD was stable in the beginning of the display period but then started to decrease towards the end of the display period. This could suggest that SOD may be more efficient as an antioxidant during the early stages of display. Therefore, even though SOD and catalase are coupled enzymes and thus would be expected to behave in a similar fashion, the evolution of the two enzyme's activities was not the same *post mortem* and there is a possibility that catalase present in meat protects SOD against inhibition of hydrogen peroxide at the beginning of the display period. Despite this difference in trend, both of the activities of SOD and catalase help to scavenge the highly-reactive superoxide and hydroxyl ion, respectively, so that any decrease in their activity may due to oxidative stress *in vivo* (Pigeolot *et al*., 1990). Anton (1993) also noted that the radical attack was almost completely inhibited when both SOD and catalase were added together.Tables 4.4 and 4.5 show that there was a significant correlation between SOD activity and a^* values.

4.1.1.4 GSHPx

In Table 4.1, the only significant difference observed was for treatment effect with the activity of GSHPx was higher in ground pork than in fresh sausages (Table 4.3). This is supported by Renerre *et al.* (1996) who stated that GSHPx activity did not significantly differ among different beef muscles and with time. However, these results contradict Hernandez *et al.* (2002), who observed that GSHPx activity decreased in both red and white muscles during storage at 4°C for 0, 2 and 4 days (wrapped with polyvinyl chloride film). Lee *et al.* (1997) also showed a similar decreasing pattern of GSHPx activity in pork muscles after 10 week storage at -15°.

4.1.1.5 Total antioxidant capacity (TEAC)

The total antioxidant capacity for both ground pork and fresh sausages was decreased significantly during the display period (Tables 4.1 and 4.3). However, there was no significant relationship between total antioxidant capacity with a* values (Tables 4.4 and 4.5). Therefore, the total antioxidant capacity of both the ground pork and fresh sausage could not be correlated with the colour stability as in the case for catalase and SOD. Renerre *et al.* (1996) stated the increase of free iron in beef during storage may induce protein oxidation which can lead to the destruction of antioxidant enzymes so they can not perform their function as free-radical scavengers as the storage period increases. Their result clearly reflects the findings of this study, where the TEAC decreased over time.

It is important to note that TEAC only measures the radical scavenging capacity of meat by inhibiting the autoxidation of $ABTS^{+}$ and thus does not indicate the reducing potential of the sample. Each antioxidant has its own mechanism in inhibiting the oxidation process (Gatellier *et al.*, 2004). Accordingly, an estimation of global antioxidant status rather than single measurement of antioxidant capacity should be used to assess the capacity of muscle to resist oxidation. Therefore, multiple methods must be used to get a better idea of antioxidant status in biological systems.

Table 4.1 The *p* values for antioxidant capacity, colour and microbial count analyses during a five day display period at constant 4ºC (three batches)

	p values		
Variables	trt	day	trt^{x}day
Catalase activity (U/g meat)	0.0001	<0.0001	0.1397
GSHx activity (U/g meat)	0.0023	0.3161	0.1255
SOD activity (U/g meat)	0.0155	<0.0001	0.6526
TEAC (mmole Trolox/L)	0.0010	<0.0001	0.9243
Relative amount of Metmyoglobin	0.0590	<0.0001	<0.0001
L* values	0.5649	0.0001	0.5717
a* values	<0.0001	<0.0001	0.7554
b* values	<0.0001	<0.0001	0.1225
Total microbial count (log_{10} (CFU)/g)	0.1288	<0.0001	0.4286
B. thermospacta microbial count (log_{10} (CFU)/g)	0.7763	<0.0001	0.1847
Lactic acid microbial count (log_{10} (CFU)/g)	0.1334	<0.0001	0.3796

SEM= standard error of mean.

Table 4.2: The means for antioxidant analyses for treatment effect of ground pork and fresh sausages during a five day display period at 4°C (three batches)

	Fresh sausages		
Antioxidant enzymes	Ground pork	Fresh pork sausage	SEM
Catalase (U/g meat)	194[a]	164[b]	4.480
GSHx (U/g meat)	0.825 [a]	0.680 [b]	0.0388
SOD (U/g meat)	45.9 [a]	37.8[b]	2.108
TEAC (mmole Trolox/L)	0.016 [a]	0.014 [b]	0.0003

Means with the same letter in the same row is not significantly different ($p<0.05$).
The multi-treatment comparisons were made using the pdiff method. SEM= standard error of mean

Table 4.3: The means for antioxidant analyses for day effect of ground pork and fresh sausages during a five day display period at 4°C (three batches)

	Catalase (U/g meat)		SOD (U/g meat)		TEAC (mmole Trolox/L)	
Day	Mean	SEM	Mean	SEM	Mean	SEM
1	281[a]	14.34	47.8[a]	2.074	0.017[a]	0.0002
2	223[b]	7.567	44.4[ab]	1.997	0.016 [b]	0.0003
3	154[c]	4.406	42.8[bc]	1.745	0.015 [c]	0.0003
4	140[d]	5.181	40.2[c]	1.484	0.014 [d]	0.0003
5	96.1[e]	7.555	34.1[d]	1.247	0.013 [e]	0.0003

Means with the same letter in the same column are not significantly different ($p<0.05$).
The multi-treatment comparisons were made using the pdiff method. .SEM= standard error of mean.

Table 4.4: Correlation coefficients of chemical, colour and microbial analyses for ground pork during a five day display period at 4°C (three batches)

	Catalase	GSHPx	SOD	TEAC	MetMb	L*	a*	b*	TSA	STAA
GSHPx	-0.35									
SOD	0.62*	0.19								
TEAC	0.65*	-0.65*	0.03							
MetMb	0.38	0.23	0.55*	-0.16						
L*	-0.19	-0.73*	-0.12	0.50*	-0.60					
a*	0.66*	0.09	0.67**	0.26	0.68**	-0.44				
b*	0.56*	-0.12	0.18	0.36	0.58*	-0.50*	0.84***			
TSA	-0.87***	0.14	-0.76**	-0.34	0.55*	0.34	-0.86***	-0.73**		
STAA	-0.93***	0.26	-0.73*	-0.35	-0.57*	0.35	-0.82***	-0.72**	0.97***	
MRS	-0.92***	0.13	-0.71*	-0.31	-0.54*	0.42	-0.81***	-0.73**	0.97***	0.96** *

* $p<0.05$ ** $p<0.01$ *** $p<0.001$

Table 4.5: Correlation coefficients of chemical, colour and microbial analyses for fresh sausages during a five day display period at 4°C (three batches)

	Catalase	GSHPx	SOD	TEAC	MetMb	L*	a*	b*	TSA	STAA
GSHPx	0.18									
SOD	0.53*	0.41								
TEAC	0.74**	-0.12	0.19							
MetMb	0.35	-0.42	-0.28	0.59**						
L*	0.27	-0.49	-0.50	0.63**	0.57**					
a*	0.80**	-0.03	0.66**	0.06	-0.02	-0.59**				
b*	0.77**	-0.22	0.43	0.32	0.27	-0.28	0.92***			
TSA	-0.78**	-0.15	-0.69**	-0.39	-0.02	0.34	-0.86***	-0.80***		
STAA	-0.84***	0.03	-0.48	-0.45*	-0.12	0.32	-0.87***	-0.85***	0.91***	
MRS	-0.83***	-0.23	-0.75**	-0.30	0.07	0.42	-0.89***	-0.80***	0.98***	0.92***

* $p<0.05$ ** $p<0.01$ *** $p<0.01$

4.1.2 Colour analyses of pork patties

4.1.2.1 Relative amount of metmyoglobin

While the values for the relative amount of metmyoglobin was significantly different on days 1 and day 5, the treatment effect was not significant (Table 4.1). There was also an interaction between treatment effect and time effect (Tables 4.1 and 4.6). In this study, a significant elevation in the amount of metmyoglobin was observed from day 2 to day 3 and from day 3 to day 4 (Table 4.7). The elevation of the amount of metmyoglobin during storage *post mortem* may be caused by both the reduction of metmyoglobin reductase activity (Zhu and Brewer, 1998) and oxygen consumption rate (Atkinson and Follet, 1973). Therefore, as time increased, the metmyoglobin accumulated so that a more pronounced grey-brown colour was observed on the surface of both the ground pork and fresh sausages. McKenna *et al*. (2005) stated that low metmyoglobin values (~1.40-1.30) were observed on day 1 for all muscles in beef. In this study, the metmyoglobin values on day 1 for both ground pork and fresh sausages was not as high as the value that was reported by McKenna *et al*. (2005) because pork has lower amount of myoglobin than beef.

When meat starts to loss its colour, the meat become less red which mean that a* values decrease and the relative amount of metmyoglobin increases. Metmyoglobin formation has been shown to negatively correlate with a* due to oxidation of myoglobin during meat storage (Renerre *et al.*, 1996). In my research, this observation was seen in ground pork (Table 4.4).but not in fresh sausages (Table 4.5).

Stewart *et al.* (1965) established a linear relationship between relative amount of metmyoglobin and K/S ratio for metmyoglobin from a mixture of beef and pork cuts. They reported that ratio values of 1.40, 1.30, 1.20, 1.10, 1.00 and 0.90 corresponded with approximately 0%, 12%, 23%, 34%, 46% and 59% metmyoglobin, respectively. Moreover, consumers begin discriminating against beef steaks when approximately 20% metmyoglobin is present (Renerre and Labas, 1987). According to Hood (1975), who used a linear relationship established by Stewart *et al.* (1965), consumer discrimination against beef steak occurs at a ratio between 1.20 and 1.24, depending on initial ratio values. In this study, however, the K/S ratio at day 1 was only 1.07 (Table 4.7) which has the highest a* values (Table 4.7). Therefore, the established value for % metmyoglobin for beef is somewhat different from the pork used in this study. The correspondence of the K/S values and % metmyoglobin in pork must be established, along with the colour sensory analysis as determined by panelists, in order to find the discrimination point of % metmyoglobin.

However, extrapolation from the results of previous studies with beef can be used to find the consumer discrimination point in this study. Hood (1975) reported that consumers could detect the difference in beef colour by 0.16 units or a 20% increase in metmyoglobin. Again, this value was established for beef, but then if the initial value of metmyoglobin at day 1 for beef (1.30, McKenna *et al*., 2005) is compared with pork sausages (1.07) as found in this study (table 4.7), the consumer could be expected to be able to discriminate a change in product colour in fresh sausages at a K/S ratio of 0.13 [(1.07/1.30) x 0.16]. Therefore, consumers should start to be able to discriminate the colour difference in ground pork pork patties in this study from day 1 to day 5 because

the K/S value was 0.14 which is higher than 0.13 (Table 4.7) but not in fresh sausages since the difference in K/S ratio from day 1 to day 5 was only 0.09 < 0.14 (Table 4.1). This result suggests that the fresh sausages had a longer shelf-life in comparison to ground pork.

Salting could decrease the ability of the endogeneous antioxidant enzymes to control superoxide anion and peroxide concentration (Kanner *et al*. 1991), thus the salted sausages would tend to discolour more vigorously than the ground pork. However, this result was not observed in this study, suggesting that lemon powder or sodium erythorbate might have a colour enhancing effect that reduced the prooxidant activity of salt.

Table 4.6 The effects of treatment and day on relative amount of myoglobin during a five display period at 4°C (three batches)

		Display time					
Variables	Treatment	day 1	day 2	day 3	day 4	day 5	SEM
Relative amount of Metmyoglobin	Ground pork	1.01	1.04	1.02	1.00	0.870	0.2904
	Fresh pork sausage	1.13	1.06	1.03	0.993	1.04	

SEM= standard error of mean.

Table 4.7: The means for colour analyses for day effect of both ground pork and fresh sausages during a five display period at 4°C (three batches)

	Relative amount of Metmyoglobin		L*values		a*values		b*values	
Day	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
1	1.07^{a}	0.0164	58.8^{a}	0.3182	20.1^{a}	0.2951	20.4^{a}	0.1245
2	1.05^{a}	0.0188	58.5^{b}	0.2637	19.4^{b}	0.1878	20.0^{b}	0.1245
3	1.02^{b}	0.0217	58.4^{b}	0.3106	18.9^{c}	0.2035	19.7^{c}	0.1245
4	0.998^{c}	0.0225	58.4^{b}	0.2933	18.3^{d}	0.1815	19.5^{d}	0.1245
5	0.956^{c}	0.0165	58.1^{c}	0.2563	17.5^{e}	0.1689	19.1^{e}	0.1245

Means with the same letter in the same column are not significantly different ($p<0.05$).
The multi-treatment comparisons were made using the pdiff method. SEM= standard error of mean

4.1.2.2 L* values

The L* values of the colour measurement of the pork patties were significantly different over time (Table 4.1); while the ground pork had higher L* value than the fresh sausages, this different was not significant (Table 4.1). Seyfert *et al.* (2007) indicated in their study that time had a predictable effect as colour stability during display where generally the L* values increased during storage, the a* values decreased during storage while b* values increased from day 1 to day 3. Then, it was followed by only a slight change from day 3 to day 6 with no pattern. This observation was also supported by Lindahl *et al.* (2006a). On the other hand, a decrease in lightness (L*) was observed throughout the storage period in different atmosphere as studied by Viana *et al.* (2005). In this study, as indicated in Table 4.7, overall the L*values tended to be rather stable in beef steaks from different muscles at 2±2 ºC. This result also agrees with McKenna *et al*. (2005) who concluded that changes in L* values were very subtle over a typical retail display time course. Bradford *et al.* (1993) similarly stated that L* values and b* values were not affected by the storage time on fresh sausages at 5-7ºC.

4.1.2.3 a* values

Both treatment and day effect were significant during the whole display period (Table 4.1). Ground pork had lower a* values over the period of day 1 to day 5 than did fresh sausages, and that this difference was significant (Table 4.8). The redness of the meat decreased over the display period and this may due to the autoxidation of the myoglobin to metmyoglobin which was clearly reflected by the elevation of the relative

content of metmyoglobin throughout the display period. The a* values significantly correlated with b* values for both the ground pork (0.84) and lemon (0.92) patties, as seen in Table 4.4 and 4.5, respectively. The positive b* values indicate yellowness in meat colour, and this yellowness had a strong relationship with the redness in this study.

Consumers are supposedly able to perceive a difference in a* value of 0.6 – 0.9 depending on the light source (Zhu and Brewer, 1999). If the consumers are able to discriminate the discolouration when a difference in a* values is equal to 0.9, then the consumers would detect the discolouration at day 2 for ground pork and at day 3 for fresh sausages in this study (Table 4.1). This result thus suggests that fresh sausages had a longer shelf life than ground pork.

Table 4.8: The means for colour analyses for treatment effect of both ground pork and fresh sausages during a five display period at 4°C (three batches)

Colour measurement	Ground pork	Fresh pork sausage	SEM
a*values	21.2[a]	16.5[b]	0.2493
b*values	20.3 [a]	19.2 [b]	0.0003

Means with the same letter in the same row is not significantly different ($p<0.05$).
The multi-treatment comparisons were made using the pdiff method. SEM= standard error of mean

4.1.2.4 b* values

As seen for a* values, the effect of treatment and time effect on b* values were significant (Table 4.1). Whereas the b* values significantly decreased every day (Table 4.7), the data clearly showed that the b* values for fresh sausage was higher than ground pork at each time (Table 4.8), As mentioned previously, Seyfert *et al.* (2007) indicated b* values increased from day 1 to 3. Moreover, Bradford *et al.* (1993) also stated that L*

values and b* values were not affected by the storage time on fresh sausages at 5-7ºC. Their result is in contradiction with this study, where the b* values decreased during the display period from day 1 to day 5. While there is a still a lack of consensus with respect to b* value, it is important to note that the majority of studies have been conducted using beef.

4.1.3 Microbial analyses

Fresh sausages are perishable products because they provide a good environment for microorganisms to grow in terms of their pH and A_w. Fresh sausages usually have a pH value not lower than 5.5 and A_w equal to or greater than 0.97 (Cocolin *et al.*, 2004). Therefore, fresh sausages provide a relatively good environment for microbial growth. Fresh sausages undergo no fermentation, drying or thermal processing so during the storage at 4°C, hygienic quality of the raw materials are critical factors affecting the final value of the products (Cocolin *et al.*, 2004). The only hurdle to spoilage of this class of product is refrigeration, and a shelf life of 10 days can be expected when these products are stored at 4°C (Cocolin *et al.*, 2004). It is clearly very important to minimize the initial bacterial load in fresh sausages during preparation stage.

In fresh sausage, the spoilage point is reached at a microbial load of 7-8 $\log_{10}$ CFU/g (Lambert *et al.*, 1991). At 10°C, spoilage occurs after 6 days, whereas at 0°C, spoilage is delayed to more than 3 weeks (Lambert *et al.*, 1991). Therefore, in this study, the shelf-life of the fresh sausages should be more than 6 days since they were displayed

at 4°C. The total microbial count only reached a maximum of 6.6 $\log_{10}$ CFU/g in ground pork by day 5 (Table 4.9).

Table 4.1 shows that the number of organisms which grew on TSA, STAA and MRS plates were significantly (p<0.05) different over time. There was no significant difference between the ground pork and fresh sausages (Table 4.1); eventhough.the ground pork patty had higher microbial count than fresh sausages. The growth rate for all 3 different microbial counts during storage period at -1°C from the production day to day 0 was slower in comparison to the growth rate during display period at 4°C from day 1 to day 5. This result suggests that the microbial growth is faster at higher temperature.

In general, meat that is stored under aerobic conditions will be spoiled more rapidly by Enterobacteriacea, lactic acid bacteria, *Pseudomonas spp*., and *Brochothrix thermosphacta* (Borch *et al.*, 1996). Specifically, *B. thermosphacta* and coliforms have been found to be the predominant bacteria associated with the spoilage of pork under all temperatures and atmospheres. *Pseudomonas spp*. only dominates under aerobic atmospheres (Liu *et al.*, 2006). In spoiled meat, *Lactobacillus sakei/curvatus* and *Leuconostoc mesenteroides* are example of lactic acid bacteria, whereas *Enterobacter amnigenus* and Hafnia are examples of Enterobacteriaceae (Borch *et al.*, 1996). When microorganisms grow in the same environment, they definitely interact with each other and their interactions during storage can play an important role in spoilage.

In the presence of Enterobacteriaceae and Pseudomonas, *B. thermosphacta* showed the same growth trends as when it was cultured alone (Russo *et al.,* 2006). However, in the presence of lactic acid bacteria, *B. thermosphacta* strains tended to yield lower counts after 5 days of incubation (Russo *et al.*, 2006). Table 4.9 shows that the

total microbial count was higher than lactic acid bacterial counts, which in turn were higher than *B. thermosphacta* counts; this trend was evident in both ground pork and fresh sausages. This result suggests that lactic acid bacteria were the dominant microorganisms in both ground pork and fresh sausages, as they made up the majority of the total microbial counts. This is also in agreement with Holley *et al.* (2004), who showed that after 48 hours the *B. thermosphacta* counts were 2 log cycles lower in the presence of lactic acid bacteria than without the lactic acid bacteria. These statements are also supported by Russo *et al.* (2006) who found that the lactic acid bacteria counts were higher than *B. thermosphacta* counts during the whole incubation period. In contrast, Cocolin *et al.* (2004) showed the growth of *Leuconostoc mesenteroides* and *B. thermosphacta* remained high until at the end of the monitoring period and they concluded these organisms were among the most active populations during their study.

This antagonistic activity of lactic acid bacteria toward *B. thermosphacta* may be due to the production of acid by the lactic acid bacteria; these organisms are known to lower the pH of the environment, as well as compete for the same substrate for growth (Greer and Dilts, 1994). It is thought, in this case, that the production of organic acid by lactic acid bacteria might be balanced by the end products of the metabolism of enterobacteria and fecal enterococci, such as ammonia as well as free amino acids produced by proteolytic activity of yeast species.

Other reasons for the inhibition of *B. thermosphacta,* besides the drop in pH during display period, include an antagonistic effect from lactic acid bacteria, whose rapid growth result in consumption of the same nutrients required by *B. thermosphacta*. Alternatively, Gardner (1981) suggested that the production of H_2O_2 by lactic acid

bacteria may be the reason for *B. thermosphacta* inhibition. Lactic acid bacteria lack peroxidase (Kono and Fridovicj, 1983), so in the presence of lactic acid bacteria under aerobic conditions, hydrogen peroxidase could accumulate and inhibit the growth of *B. thermosphacta*. It is important to note, however, that even though the growth of the lactic acid bacteria is very rapid, they did not contribute significantly to meat spoilage because of reduced secretion of metabolites with less offensive characteristics than those produced by aerobic bacteria such as *B. thermosphacta* (Viana *et al.*, 2005).

In fresh sausages, all of the counts on TSA, STAA and MRS (-0.86, -0.87, -0.89, repectively) had a significant negative correlations only with a* values (Table 4.5). Moreover, in ground pork, the microbial counts significantly correlated with the relative amount of metmyoglobin and a* values (Table 4.4). In general, it can be concluded that microbial growth caused meat discolouration in terms of lowering a* values and increasing the relative amount of metmyoglobin during the display period.

Aerobic bacterial growth can accelerate oxidation of meat pigments that can lead to meat discolouration (Lawrie, 1991) by increasing the rate of myoglobin autoxidation in aerobic conditions (Renerree, 2000) through the elevation of oxygen consumption and the reduction of partial oxygen pressure to the level critical for myoglobin oxidation (Cheah and Ledward, 1997). A reduction in oxygen partial pressure (~10 mm Hg) on the meat surface as a result the microbial growth over time could cause discolouration on meat surface due to the accumulation of metmyoglobin that would be formed more rapidly under lower oxygen partial pressure (Labadie, 1999).

Microorganisms also have the ability to change the meat pH and produce amino acids and amines through proteolysis and glycolysis that further induce the myoglobin

autoxidation (Hedrick *et al*., 1989). Thus, any factors that can enhance microbial growth will also increase of the rate myoglobin oxidation rate (Sofos *et al*., 2000) which then leads to meat discolouration. However, it is important to note that anaerobic bacteria do not generally cause meat discolouration (Kropf *et al*., 1986) and lactic acid bacteria have no influence on development of brown colour (Sofos *et al*., 2000).

Table 4.9: The means for microbial analyses for day effect of both ground pork and fresh sausages during display period a five at 4°C (three batches)

	Total microbial count (log_{10} CFU/g)		*B. thermospha*cta count (log_{10} CFU/g)		Lactic acid bacteria count (log_{10} CFU/g)	
Day	Mean	SEM	Mean	SEM	Mean	SEM
5	6.1^{a}	0.243	5.508^{a}	0.336	6.2^{a}	0.277
4	6.0^{b}	0.227	4.776^{b}	0.328	5.6^{b}	0.277
3	5.0^{c}	0.350	4.046^{c}	0.207	5.0^{c}	0.277
2	4.2^{d}	0.313	3.266^{d}	0.265	4.2^{d}	0.277
1	3.7^{e}	0.256	2.496^{e}	0.363	3.6^{e}	0.277

Means with the same letter in the same column are not significantly different ($p<0.05$)
The multi-treatment comparisons were made using the pdiff method. SEM= standard error of mean

4.1.4 Redox potential analysis

Redox potential (E_h) is a measurement of the ease by which a substance gains or loses electrons. E_h is measured in millivolts. A fully-oxidized standard oxygen electrode will have an E_h of +810 mV at pH 7.0, 30° C, and under the same conditions, a completely reduced standard hydrogen electrode will have an E_h of –420 mV. The E_h is dependent on the pH of the substrate; normally, the E_h is taken at pH 7.0 (Reichart *et al.*, 2006).

Different groups of meat products have different redox potential values. The E_h of raw meat is in the negative range, roughly around -200 to -300 mV (Rödel and

Scheüer, 1999b). Meat products, however, have a completely changed redox system compared to raw meat. Meat processing and the addition of ingredients and additives to meat products shift the redox to a more positive range; for example, fermented raw meat products have E_h values ranging between 100 to 200 mV, and heated meat products have E_h values ranging between -25 to 100 mV (Rödel and Scheüer, 1999b). As indicated in Figure 4.1 and Figure 4.2, the E_h values on the processed pork patties were on the positive side (~ 200±50 mV) initially, due to the grinding process that incorporates O_2 in the meat system. *Post mortem* meat redox potential decreases very rapidly from initial values of 100 mV down to -100 mV or – 200 mV (Ahn and Maurer, 1989). This reduction was seen in this study but the reduction did not exceed – 50 mV (Figure 4.2).

Many extrinsic factors influence microbial growth, including the oxidation-reduction potential or E_h (Tabatabai and Walker, 1970; Motilva *et al.*, 1992; Barnes and Ingrams, 1956). Therefore, the measurement of redox potential the meat products could be intended to assess microbial activity that also related to colour stability of the meat product. According to Kukec *et al.* (2002), redox potential measurement could assess the ability of life of microorganisms, growth as well as the physiological activity in defined environment. Indeed, it is very important to know the parameters that influence the survival and growth of microorganisms in the products to predict the shelf-life of meat products (Rödel and Lücke, 1989). Microbial growth that caused spoilage usually occurs on the surface of meat products, and E_h on the surface of the meat is more readily affected by the microbial activity than the E_h in the middle of the meat products.

There was a significant negative correlation of the redox potential to the total count of microorganisms and the distinctively lower potential values after storage of 14

days in samples with visible sign of spoilage (Hoffman, 1974). In this study, as seen in Figure 4.1 and 4.2, the E_h on the surface of the both patty treatments decreased more significantly nearer to the end of display period; this reduction of redox potential value likely reflects the more rapid growth of microbial at this time. Kukec *et al.* (2002) also indicated that exponential phase growth was followed by a fast drop of redox potential.

Benedict *et al.* (1975) noted that the significant decrease of both pH and E_h after 8 and 10 days of storage may have been caused by an alteration of bacterial metabolism. In general, the energy source for microbial growth is via the biological oxidation of complex macromolecules in meat, a process that results in the formation of a more reducing environment. Metabolic oxygen consumption further contributes to this effect and also forms reducing compounds in the surrounding microbial microenvironment (Reichart *et al.*, 2007). Metabolites resulting from bacterial activity are generally water soluble and would affect the pH and E_h effectively.

The shape of the redox potential curve is characteristic of the type of microorganisms numerically dominant during bacterial growth (Reichart *et al.*, 2007). Tabatabai and Walker (1970) stated that initial E_h and dissolved oxygen content of the culture medium played an important role in microbial growth. In their study, they showed that pure cultures of *Clostridium perfringens* and *Pseudomonas fluorescens* grew better in media that had an initial E_h of 200 mV (with oxygen present) than in the media that had initial E_h of 40 mV (without oxygen present). Barnes and Ingram (1956) demonstrated that *Cl. perfringens* only initiated growth in fresh meat after the E_h value dropped to below -36 mV. In contrast, Rödel and Lücke (1989) did not observe any effect of initial E_h on the growth of *Bacillus subtilis* and *Basillus licheniformis.*

Regardless, most studies show a decrease in redox potential during exponential growth of various bacteria under both anaerobic and aerobic conditions (Tabatabai and Walker, 1970). As far as this study is concerned, the later statement can be concluded, but any further interpretation regarding the optimum initial E_h could not be derived.

Gradients of E_h may exist from the surface to the center of the foods when inward gaseous diffusion is restricted (Brown and Emberger, 1980). Therefore, in the center of the patty, a more anaerobic environment exists than on the surface of the meat patty, usually reflected by a more negative E_h. In this study, the pork patties were only 10 mm in thickness, so the difference in the E_h tended to be not as noticeable. The penetration depth depends on the rate of oxygen diffusion, rate of oxygen consumption and the oxygen partial pressure (O'Keeffe and Hood 1982; Renerre 2000). *Post mortem*, meat continues to consume oxygen for the mitochondrial electron transport chain. Therefore, myoglobin must compete with mitochondrial respiratory system for oxygen that diffuses into the meat from the atmosphere. The oxygen consumption rate (OCR) decreases over time because of the depletion of substrate coenzymes (NADH) and the degradation of enzymes involved in mitochondrial respiration (O'Keeffe and Hood 1982).Therefore, besides microbial activity, redox potential can also serve as an indicator of mitochondrial respiration *post mortem* during the display period through the redox measurements from microelectrodes that were located in the center of the patties. When the OCR decreases in meat patties, the E_h should increase since more oxygen is now available, as seen in Figure 4.2. This elevation of redox potential at the end of the display period may indicate that more oxygen has become available and this oxygen now can bind myoglobin to form oxymyoglobin in fresh sausages.

Difficulties arise in obtaining accurate redox measurements because it is hard to obtain equilibrium in redox at the point of measurement. The irregularity of the E_h profile and the variations between measurements in the fresh sausages may also be due to the heterogeneity of texture of the sausages (FDA, 2001); thus, it is hard to obtain reproducible E_h results among replicates. These values can be highly variable depending on changes in the pH of the food, microbial growth, packaging, partial pressure of oxygen in the storage environment, and ingredients and composition, such as, protein, ascorbic acid, reducing sugars and oxidation level of cations. Therefore, in order to a get consistent measure of absolute redox potential, the affecting variables must be kept constant. Another important factor is the poising capacity, or buffering capacity, of the food system; this factor influences the extent that a food resists external affected changes in E_h (FDA, 2001). The poising capacity of the food will be affected by oxidizing and reducing constituents in the food as well as by the presence of active respiratory enzyme systems. Lastly, irregularities in the E_h measurement may also be affected by volatile components that affect the redox potential in the process before reaching the equilibrium (H_2S, H_2, O_2 and SO_2). Therefore, it is somewhat difficult to get reliable absolute redox potential values, thus if E_h measurements are not performed in combination with other measure of microbial growth potential, incorrect conclusions can be derived. Even though the absolute redox potential value is hard to measure, in this study, the absolute redox values are not needed in order to observe the trend of the redox values during storage.

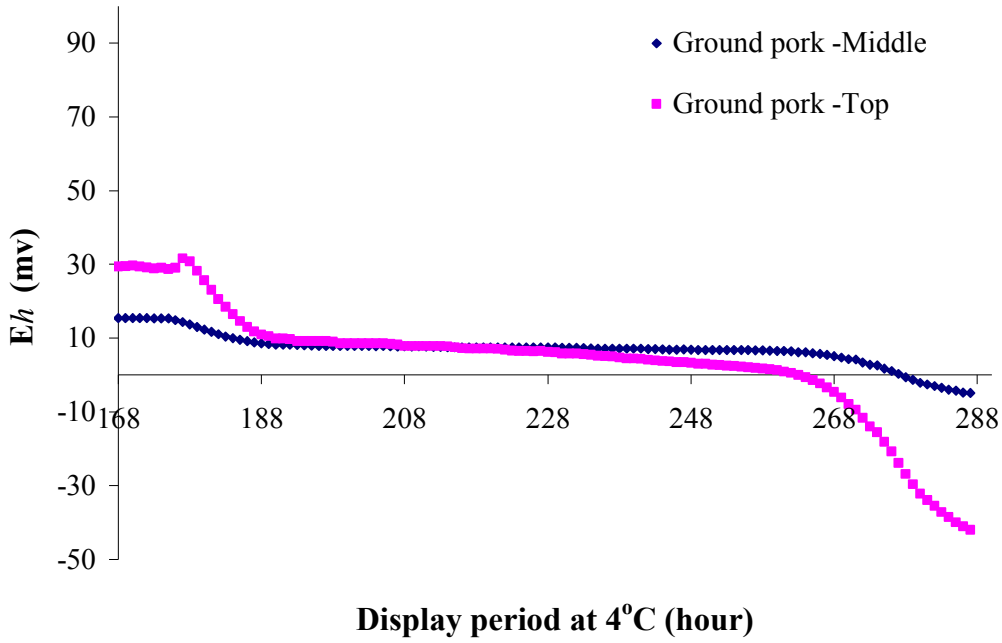


Figure 4.1: The effect of storage period at -1°C and display period at 4°C (illuminated with 850 lux – 1100 lux light) on the redox potential (Eh) of ground pork with microelectrodes positioned 2 mm from the patty surface (Top) and 5 mm from the patty surface (Middle), starting from day 1 (168 hours) to day 5 (288 hours) of the display period.

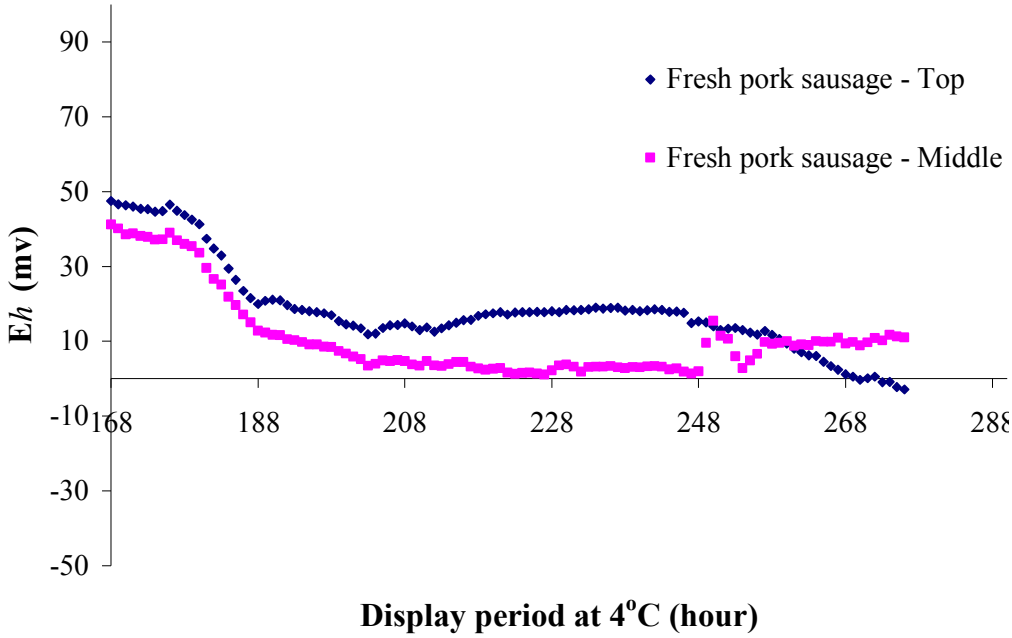


Figure 4.2: The effect of storage period at -1°C and display period at 4°C (illuminated with 850 lux – 1100 lux light) on the redox potential (Eh) for fresh sausages with the microelectrodes positioned 2 mm from the patty surface (Top) and 5 mm from the patty surface (Middle), starting from day 1 (168 hours) to day 5 (288 hours) of the display period

4.1.5 Proximate analysis and pH measurement of meat patties

In this study, proximate analyses were done for controlin the variation of the raw picnic shoulder from the suppler. In this study, the fat content of each sausage batch was kept constant at around in order to minimize variation in terms of the source of radical that could affect the efficacy of the endogenous antioxidant enzymes and colour stability. As shown in Table 4.10, the protein content had a 6.7% coefficient of variation.

Therefore, the variability among batches was small which was not the source of much variation.

In figure 4.3, day -7 represents the production day (slaughter), and the pH at this point was lower than the other days. Kalliopi *et al.* (2005) reported that the pH in the fresh sausages had an initial value of 5.61± 0.007 and did not change significantly during the display period. As shown in Figure 4.3, the pH values of ground pork were higher than the fresh sausages. Over time during both storage and display, the pH of fresh sausages did not really change, whereas the pH values for ground pork were seen to increase rapidly, especially from day 4 to day 5 of the display period.

Table 4.10: The means, standard deviation and coefficient of variation (CV) of proximate analysis of the picnic shoulder boneless pork obtained from Maple Leaf Foods, shown for three different batches

	Proximate analysis		
	Moisture (%)	Fat (%)	Protein (%)
Batch 1	65.5	14.6	16.9
Batch 2	64.1	16.6	16.2
Batch 3	65.5	15.1	18.4
Average	65.0	15.4	17.2
STD	0.8	1.0	1.1
CV (%)	1.2	6.7	6.5

STD = standard deviation, CV = coefficient variation

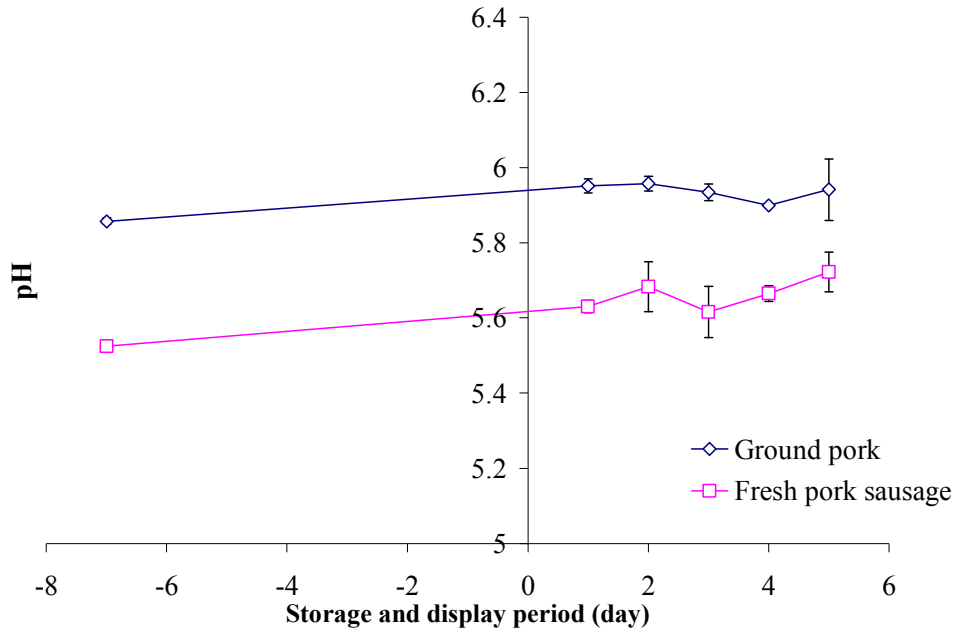


Figure 4.3: The pH values of both ground pork and fresh sausages during the storage and display period (4°C and illuminated with 850 lux – 1100 lux light) at constant 4°C (three batches)

4.2. Study 2 - Assessing the effect of non-meat ingredients on the stability of fresh sausage over time at 4°C

4.2.1 Antioxidant activity

As indicated in Table 4.11, the day effect was found to be significant for catalase, GSHPx and SOD activities for all the types of fresh sausage (A, B, C and D), with decreases in the respective values over time (Table 4.12). As known, the oxidative stability of muscle foods can be altered by food processing operations and food

additives. In general, enzyme activities are affected by electrolytes and ionic strength (Richardson and Hyslop, 1985). Maximal inactivation of catalase, glutathione peroxidase and superoxide dismutase in stored salted ground pork were 2% NaCl, 0.5 and 1.0% NaCl and 1.0% NaCl, respectively (Lee *et al*., 1997). However, the addition of other ingredients, such as sodium erythorbate and lemon juice powder, as part of the fresh sausages formulation could increase the oxidative stability of the meat products. Coronado *et al*. (2002) concluded that the addition of sodium erythorbate in sausage's formulation increased the oxidative stability of the sausages due to its antioxidant capacity. As indicated earlier, erythorbate is an isomer of ascorbate and thus erythorbate functions as a reductant in food and biological systems. Ascorbic acid promotes iron reduction and solubilization, thus preventing the formation of insoluble ferric hydroxides. Moreover, it also can replace $O_2^{\bullet-}$ which is dismutated to hydrogen peroxide and participates in the Fenton reaction (Khan and Martell, 1967).

In this study, there was no synergistic interaction between lemon juice powder and sodium erythorbate for all catalase, GSHPx and SOD activities (Table 4.11). The only significant interaction was between sodium erythorbate and catalase activity. This interaction means that the addition of 0.05% sodium erythorbate in fresh sausage B and D had a significant positive effect on catalase activity in comparison to fresh sausage A and C (Table 3.7). In Figure 4.4, the decreasing rates of catalase activity for fresh sausage A and C can be seen to be lower than that for B and D. Antioxidants need to be sacrificed to protect against oxidation. Therefore, the different decreasing rate of catalase activity in Figure 4.4 could mean that sodium erythorbate could influence how the catalase acts as an antioxidant

Table 4.11: The *p* values for: Lemon, Sodium erythorbate, Lemon x Sodium erythorbate, Day, and Lemon x Sodium erythorbate x Day

	***p* values**				
Variables	Lemon juice powder	Sodium erythorbate	Lemon juice powder x Sodium erythorbate	Day	Lemon juice powder x Sodium erythorbate x day
Catalase activity (U/g meat)	0.4341	0.0119	0.4653	<0.0001	0.2447
GSHx activity (U/g meat)	0.6992	0.1876	0.5120	<0.0001	0.8920
SOD activity (IU/g meat)	0.3867	0.9139	0.5958	0.0055	0.9674
Relative amount of metmyoglobin	0.1057	0.0986	0.4656	0.0002	0.7688
L* values	0.1489	0.0377	0.4008	0.0018	0.8919
a* values	0.256	0.0366	0.7020	0.0004	0.9469
b* values	0.3641	0.4546	0.5625	0.0015	0.9664
Chroma	0.1919	0.0514	0.9931	<0.0001	0.9958
Hue	0.5118	0.0661	0.5096	0.0015	0.8911
Total microbial count (log_{10} (CFU)/g)	0.4309	0.8582	0.9003	<0.0001	0.4749
B. thermosphacta microbial count (log_{10} (CFU)/g)	0.4117	0.9007	0.7362	<0.0001	0.7522
Lactic acid microbial count (log_{10} (CFU)/g)	0.4544	0.4730	0.5112	<0.0001	0.1525

Table 4.12: The mean all types of fresh sausages A, B, C and D for catalase, SOD and GSHPx activity during a ten day display period at 4°C for (three batches)

	Catalase activity (U/g meat)		SOD (IU/ g meat)		GSHPx (U/g meat)	
Day	Mean	SEM	Mean	SEM	Mean	SEM
-7	205 [a]	4.346	34.3 [a]	1.519	1.31 [a]	0.029
1	201 [a]	4.111	31.5 [ab]	1.519	1.28 [ab]	0.029
3	170 [b]	10.99	29.5 [bc]	1.519	1.21 [bc]	0.029
5	139 [c]	7.707	29.5 [bc]	1.519	1.16 [cd]	0.029
7	121 [d]	4.874	27.9 [bc]	1.519	1.15 [cd]	0.029
9	109 [d]	9.020	25.7 [c]	1.519	1.12 [d]	0.029

Means with the same letter in the same column are not significantly different ($p<0.05$)
The multi-treatment comparisons were made using the pdiff method. SEM= standard error of mean

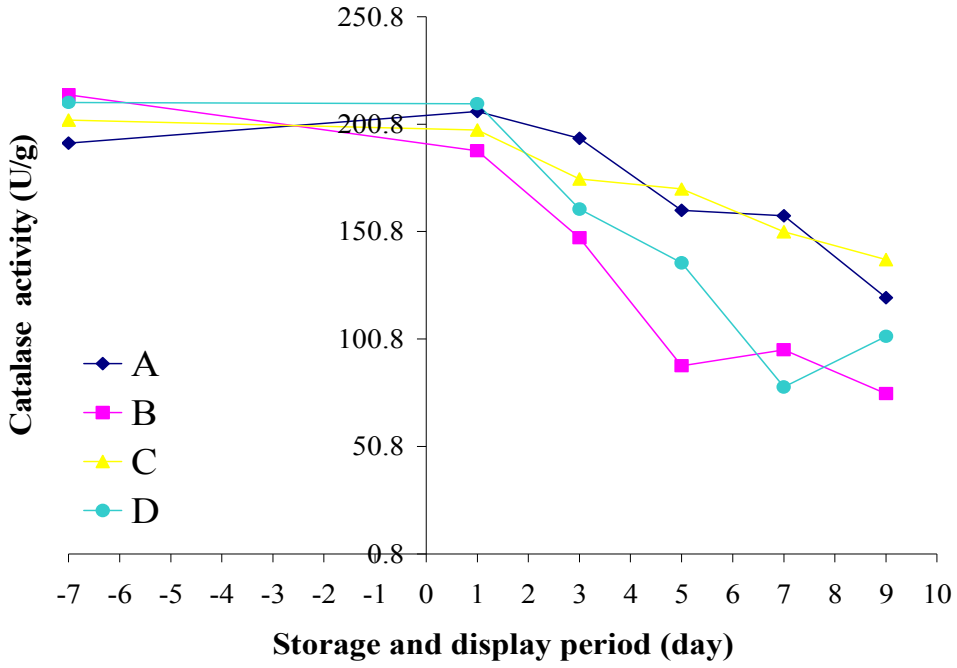


Figure 4.4: The effect of sodium erythorbate and lemon juice powder treatment (illuminated with 850 lux – 1100 lux light) on the L* values for fresh sausages A, B, C and D during storage (-1°C) and display period (4°C). A = 0% sodium erythorbate and 0% lemon juice powder, B = 0.05% sodium erythorbate, C = 0.25% lemon juice powder, D = 0.05% sodium erythorbate and 0.25% lemon juice powder

4.2.2 Colour

Fresh sausage formulation A decreased the most rapidly in terms of a* values and increased the most rapidly in terms of the relative amount of metmyoglobin oxidation (Table 4.13). This result indicates that NaCl may have increased the oxidation rate. It has previously been postulated that the prooxidant activity is associated with the ability of NaCl to alter the distribution and reactivity of iron (Decker and Xu, 1998), thereby affecting colour stability. However, the addition of sodium erythorbate and lemon juice powder in different type of fresh sausage patties could balance the negative effect of sodium chloride.

Sodium erythorbate, the less expensive salt isomer of ascorbic acid, performed similarly to ascorbic acid with respect to colour regardless of their distinct difference in pH (Mancini *et al*., 2006). Shivas *et al*. (1984) indicated that ascorbic acid is a reducing agent that could stabilize oxymyoglobin in meat products and delay lipid oxidation. In other words, it is capable of capable of maintaining myoglobin in a reduced ferrous state (Shivas *et al*., 1984). As indicated in Table 4.14, only sodium erythorbate had a significant positive effect on fresh sausages colour, particularly the L* values and a* values. The lightness of fresh sausages formulations B and D was significantly lower than A and C (Table 4.14). The addition of 0.05% sodium erythorbate also increased the redness of fresh sausage formulations B and D (Table 4.14).

Table 4.13: The mean for all the types of fresh sausage (A, B, C and D) for relative amount of metmyoglobin, L* values, a* values, b* values, chroma and hue at production day and during a ten day display period at 4°C (three batches). A = 0% sodium erythorbate and 0% lemon juice powder, B = 0.05% sodium erythorbate, C = 0.25% lemon juice powder, D = 0.05% sodium erythorbate and 0.25% lemon juice powder

	Relative amount of metmyoglobin		L* values		a* values		b* values		Chroma		Hue	
Day	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
-7	1.25 [a]	0.0028	54.8 [d]	0.3266	23.6 [a]	0.4390	20.2 [ab]	0.6106	31.1 [a]	0.7245	40.4 [e]	0.3753
2	1.08 [b]	0.0199	57.7 [bc]	0.3101	20.4 [b]	0.5272	19.9 [a]	0.2184	28.6 [b]	0.4813	44.5 [d]	0.6529
4	1.04 [c]	0.0231	57.9 [b]	0.1583	19.2 [c]	0.6328	19.7 [a]	0.2321	27.5 [c]	0.5564	45.9 [c]	0.8356
6	0.927 [d]	0.0379	57.6 [c]	0.1467	17.1 [d]	1.037	19.2 [b]	0.3387	25.8 [d]	0.8667	48.9 [b]	1.561
8	0.801 [e]	0.0266	57.7 [bc]	0.2809	13.6 [e]	0.6227	18.2 [c]	0.2432	22.8 [e]	0.4043	53.5 [a]	1.369
10	0.757 [f]	0.0201	58.5 [a]	0.2687	12.9 [e]	0.3470	12.9 [c]	0.3434	22.1 [e]	0.2278	54.4 [a]	1.126

Means with the same letter in the same column are not significantly different ($p<0.05$)
The multi-treatment comparisons were made using the pdiff method. SEM= standard error of mean

Table 4.14: The effect of sodium erythorbate on L*values and a* values for 3 sausage batches during a seven days of storage period at -1°C and a ten day display period at 4°C for fresh sausage formulations with 0.00% sodium erythorbate (formulations A and C) and with 0.05% sodium erythorbate (formulations B and D)

Sodium erythorbate	L* values		a* values	
	Mean	SEM	Mean	SEM
0.00%	57.7 [a]	0.1833	16.6 [b]	0.6678
0.05%	57.1 [b]		19.0 [a]	

Means with the same letter in the same column are not significantly different ($p<0.05$)
The multi-treatment comparisons were made using the pdiff method. SEM= standard error of mean

The reducing activity of ascorbic acid might improve muscle colour stability via metmyoglobin reduction (Lee *et al*., 1999). Even though the antioxidant activity of ascorbic acid in meat and meat products on lipid oxidation have been found to be dependent on concentration, the presence of transition metal ions, and the presence of other antioxidants (Djanane *et al.,* 2002), the addition of 0.05% sodium erythorbate had a positive effect on meat colour stability during storage and display.

In this study, the lemon juice powder that was used contained 26.3 % citric acid. Citric acid is commonly added to food substances to chelate metal ions and control pH. Moreover, it has also been shown to have effects on food color (Bouchard and Merritt, 1979). The pH of the 2% lemon juice powder solution was 2.85. Due to this low pH, it additionally reduced the pH of samples, which contributed to lightness (Sommers *et al*., 2003), a statement supported by the result in this study. According to Mancini *et al*. (2007), the effects of 1% and 3% citric acid was not as beneficial during display and 10% citric acid actually accelerated the discolouration process. In this study, only 0.25%

lemon juice powder, which contains 0.07% citric acid, was used for fresh sausage formulations C and D, and this amount was not beneficial in delaying discolouration.

The a* values of fresh sausage formulation B was higher than fresh sausage formulation C (Table 4.14). This result is in accordance with Nocolalde *et al*. (2006) who stated that at day 8, ascorbic acid-treated samples remained visibly redder than citric acid-treated samples (Nocolalde *et al*., 2006).

It has been reported that antioxidant activity of ascorbic acid is limited by the abundance of iron in meat because metal-induced oxidation of reducing agents often makes them inactive (Mancini *et al*., 2007). Thus, the addition of chelators such as citric acid would improve ascorbic acid's efficacy in muscle food products and therefore, their colour may be improved by the addition of citric acid. Lund *et al*. (2006) found that the addition of citrate in combination with ascorbate lead to the reduction of lipid oxidation in minced beef patties in the dark up to 6 days at 4ºC. In their study, after 3 days of storage only ascorbate and citrate preserved the red colour compared to no addition of antioxidant and addition of rosemary extract. However, according to Mancini *et al.* (2007) this relationship is not that straightforward since no synergistic effect was observed in their study whereas the use of only ascorbic acid was more beneficial than the combination of citric acid and ascorbic acid; an effect seen in this study (Table 4.11). Even though fresh sausage formulation D had the same low pH as fresh sausage C (Table 4.15 and Figure 4.5), the colour profile of fresh sausage formulation D was almost the same as fresh sausage B. Therefore, it is more likely that the addition of sodium erythorbate balanced out the negative effect of lemon juice powder addition on meat colour due to its low pH than the other way around. In short, since there was no

advantage in term of colour when both sodium erythorbate and lemon juice powder were added (at the concentrations used in this study), it would be more economical if meat manufacturers just added sodium erythorbate alone in their formulation at the concentration as indicated in this study.

Table 4.15: The effect lemon juice powder on pH for 3 sausage batches during a seven days of storage period at -1°C and a ten days display period at 4°C with 0.00% lemon juice powder (formulations A and B) and with 0.25% lemon juice powder (formulation C and D)

	pH	
Lemon juice powder	Mean	SEM
0.00%	6.08 [a]	0.0928
0.25%	5.83 [a]	

Means with the same letter in the same column are not significantly different ($p<0.05$)
The multi-treatment comparisons were made using the pdiff method. SEM= standard error of mean

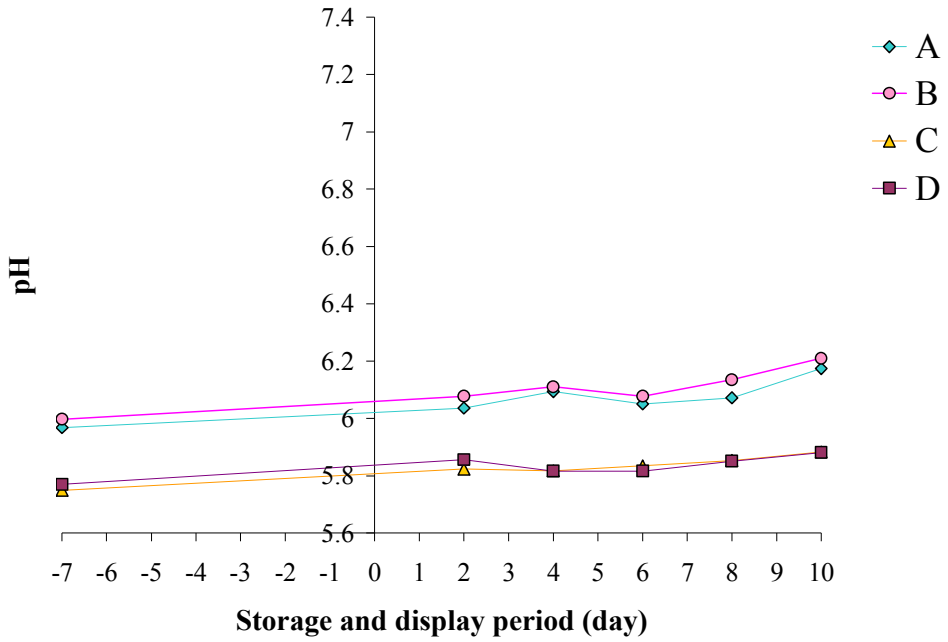


Figure 4.5: The pH values of pork patties during storage (seven days at -1°C in the dark) and display period (ten days at 4°C and illuminated with 850 lux – 1100 lux light). A = 0% sodium erythorbate and 0% lemon juice powder, B = 0.05% sodium erythorbate, C = 0.25% lemon juice powder, D = 0.05% sodium erythorbate and 0.25% lemon juice powder

4.2.3 Microbiology

Table 4.11 shows that there was no significant interaction between the non-meat ingredients and microbial count. However, there was a significant day effect on all classes of microbial counts with an increasing trend during the storage and display period (Tables 4.16). Organic acids can inhibit microbial growth and are routinely used as preservatives (Sommers *et al*., 2003). Buchanan and Golden (1994) indicated that citric acid exerted its antimicrobial effects via pH dependent- and independent-mechanisms. On the other hand, ascorbic acid did not inhibit microbial growth at various concentrations (Shivas *et al*., 1994; Soho and Anjeneyulu, 1997). In this study, neither sodium erythorbate nor citric acid exhibited antimicrobial activity in comparison to the control. Additionally, there was no synergistic effect between sodium erythorbate and citric acid (Table 4.11). This result agrees with Rhee *et al*. (1997) who concluded that citrate and ascorbate did not reduce aerobic plate counts in cooked/aerobically refrigerated beef carrageenan patties

Table 4.16: The mean of three batches (replicate) of each four formulations for total microorganisms, B. thermosphacta and lactic acid bacteria at production day and during a ten day display period at 4°C (three batches)

	Total microbial count (log_{10} (CFU)/g)		*B. thermosphacta* microbial count (log_{10} (CFU)/g)		Lactic acid microbial count (log_{10} (CFU)/g)	
Day	Mean	SEM	Mean	SEM	Mean	SEM
-7	4.0[f]	0.194	1.9[e]	0.088	3.5[f]	0.045
2	4.7[e]	0.137	2.6[d]	0.089	4.0[e]	0.046
4	6.1[d]	0.311	4.0[c]	0.163	5.0[d]	0.081
6	7.3[c]	0.299	5.5[b]	0.316	6.0[c]	0.075
8	8.3[b]	0.149	6.0[a]	0.317	6.6[b]	0.106
10	8.8[a]	0.175	6.4[a]	0.415	7.4[a]	0.161

Means with the same letter in the same column are not significantly different ($p<0.05$)
The multi-treatment comparisons were made using the pdiff method. SEM= standard error of mean

Microorganisms can metabolize glucose, lactic acid, certain amino acids, nucleotides, urea and sarcoplasmic proteins during storage. Microorganisms typically utilize the simpler constituents first, such as glucose, and thereafter use the more complex compounds, such as protein. In a study by Dainty (1996), a decrease in surface glucose concentration was first detected when cell numbers exceed $10^7/cm^2$, but 5 mm below the surface there was no reduction in glucose concentration. By the time bacterial numbers had reached $10^8/cm^2$, glucose was no longer detectable at the surface and decreased concentrations were detectable up to 10 mm lower. Bacteria then keep growing by utilizing various amino acids, as well as lactic acid. These secondary substrates do not initially become depleted at the surface. Shortly after this period, pH and ammonia concentrations began to increase at the surface and continue to do so until maximum cell numbers ($10^9/cm^2$) are reached (Dainty, 1996). Byproduct of protein consumption produces substances that cause spoilage in meat and meat products.

Therefore, the addition of non-meat ingredients will not affect the microbiological profile unless more carbohydrate is added into the formulation to alter the metabolism of the microorganisms. Salt and sodium erythorbate do not contain any glucose but lemon juice powder contains 91.2 g of total carbohydrate per 100 g of lemon juice powder. In this study, only 3.75 g of lemon juice powder was used in 1.5 kg batch meat batter, thus there is only 0.274 g of extra carbohydrate added in every 120 g fresh sausage patty. This amount was seemingly not sufficient to cause a significant difference in the growth of microbes in fresh sausage formulation C in comparison to fresh sausage formulations A, B and D.

4.2.4 Redox potential

Rödel and Scheuer (2000a) indicated that there is a correlation between redox potential and ingredients which are used in processing. According to their research, sodium ascorbate did not affect the pH of the sausage, but did lead to decreasing redox potential values. However, as the concentration of the sodium ascorbate reached 0.04 %, any further increase in concentration had little influence on the redox potential. In the present study, the initial redox potential in the middle of fresh sausage formulation A was 20 - 40 mV, and when 0.05% sodium ascorbate was added (fresh sausage B), the redox potential of the sausage was approximately – 40 mV- – 50 mV (Figure 4.6). Since sodium ascorbate is a reductant, this result is not unexpected. Moreover, Holownia *et al.* (2003) indicated that raw chicken containing sodium erythorbate had a more negative redox potential than did the control, which means it had greater reducing conditions than

control. Formulation D also contained 0.05% sodium erythorbate, and thus was also expected to have a low redox potential. However, as indicated in Figure 4.7, the redox potential of fresh sausage D was not as low as the redox potential of fresh sausage formulation B. This result may suggest that the interaction of lemon juice powder, which contained 26.3% citric acid and sodium erythorbate, increased the redox potential. The high reducing conditions allowed greater reactivity and interconversion of pigments that are not desirable from the perspective of meat colour because the *in situ* levels of reductants could control rates of metmyoglobin reduction to oxymyoglobin (Antonini and Brunori, 1971) and give a better colour stability in fresh pork sausage. The result from colour analyses showed that fresh sausage with sodium erythorbate had better colour and this may be due to a lower redox environment.

Citric acid does not have any reductant capacity but does function as a transition metal scavenger or synergist. Therefore, it does not have the ability to reduce the redox potential of the system but it does lower the pH of system (Figure 4.6). Figure 4.7 indicates that even though fresh sausage formulations A and C started at different redox potentials, eventually they came down to almost the same values. Moreover, fresh sausage formulations A and C had higher redox potentials in the patty center than patties B and D. This result suggested that citric acid did not have a positive affect the redox potential of the fresh pork sausage. The higher the redox potential, the more suitable it is for microbial growth, thus fresh sausage formulations A and C were more suitable for microbial growth. Moreover, as shown in the previous section, neither the combination of 0.05% sodium erythorbate nor 0.25% lemon juice powder alone had a significant effect on microbial activity. The surface redox potential of fresh sausage formulations A,

B, C and D should behave similarly, however Figure 4.7 (A top) did not follow this trend.

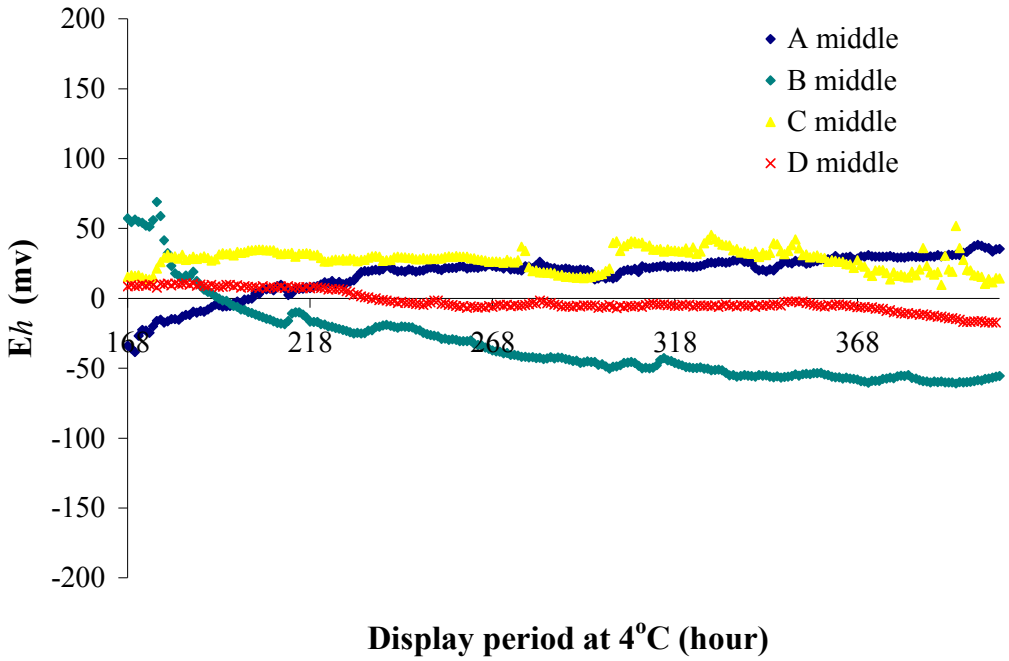


Figure 4.6: The effect of display period at 4°C (illuminated with 850 lux – 1100 lux light) on the redox potential (Eh) of fresh pork A, fresh pork B, fresh pork C and fresh pork D measured 5 mm from the surface of the patties (Middle) from day 1(168 hours) to day 10 (407 hours). A = 0% sodium erythorbate and 0% lemon juice powder, B = 0.05% sodium erythorbate, C = 0.25% lemon juice powder, D = 0.05% sodium erythorbate and 0.25% lemon juice powder

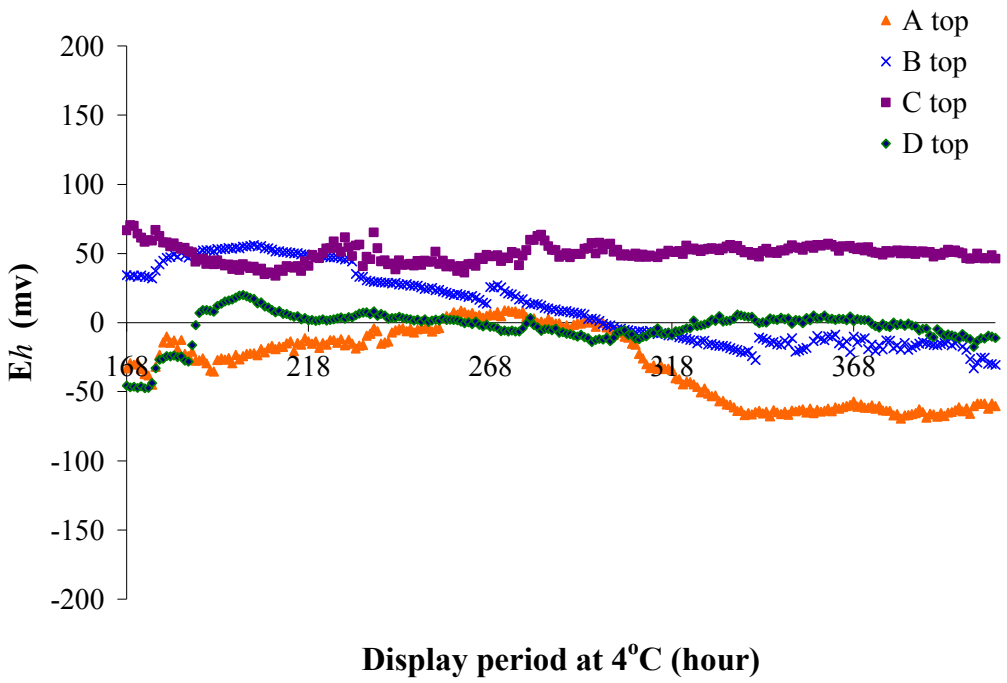


Figure 4.7: The effect of display period at 4°C (illuminated with 850 lux – 1100 lux light) on the redox potential (Eh) of fresh pork A, fresh pork B, fresh pork C and fresh pork D measured 2 mm from the surface of the patties (Top) from day 1(168 hours) to day 10 (407 hours). A = 0% sodium erythorbate and 0% lemon juice powder, B = 0.05% sodium erythorbate, C = 0.25% lemon juice powder, D = 0.05% sodium erythorbate and 0.25% lemon juice powder

4.2.5 Fresh sausages made using DFD picnic boneless pork

During the third replication (batch) of this study, DFD (dark, firm, and dry) picnic boneless pork was used to make fresh sausages due to a change in meat supplier from Maple Leaf to Country Choice Meats (Duck Lake, SK). This batch was not included in other 3 batches so the analysis of DFD pork sausage was only based on one batch so the findings regarding DFD fresh sausages were only preliminary.The DFD pork sausages in this batch had a different microbial, colour profile and catalase activity

than did the other batches. DFD meat has a higher ultimate pH (>6.0) and lower glucose content (Newton and Gill, 1981).Figure 4.8 shows that DFD fresh sausages had higher initial pH values, making DFD a better environment for microbial growth; the initial microbial counts were higher for all time points and treatments using this material (Figure 4.13). Not only that, the microbial growth was more rapid in DFD fresh sausages than in normal fresh sausages (Figure 4.9). The substantial increase of pH (Figure 4.8) at day 4 may be due to the rapid increase in microbial growth, as indicated in Figure 4.9. The growth then slowed down at day 6 in DFD fresh sausages, suggestive of entry into the stationary phase.

DFD meat has been known to spoil more rapidly than that of meat with normal pH, however the reasons are not completely clear. Newton and Gill (1981) suggested that DFD meat spoils more rapidly because it contains low concentration or even complete absence of glucose and glucose-6-phosphate in DFD meat means. This suggests that bacteria use amino acids and lactic acids are the initial substrates for growth. Consequently, DFD meat usually spoils more rapidly than normal meat. Newton and Gill (1978) compared the growth of *Pseudomonas sp.* on DFD beef with pH that was reduced with citric acid, as well as on DFD meat with glucose was supplemented to levels found in normal pH meat. Their results showed that off-odours were detected sooner and at lower microbial numbers in the samples lacking glucose regardless of pH. Therefore, the reason why DFD spoils faster is not due to microbial growth *per se*. Early spoilage is more likely due to the fact that microorganisms in DFD meat metabolize protein in earlier stage than in normal meat.

The DFD fresh sausages had higher L* values to begin with than normal fresh sausages, as indicated in Figure 4.10 b. Moreover, L* values increased up until day 4 at which time the L* values started to decrease. This trend did not happen for the normal fresh sausages (Figure 4.10 a). The DFD and normal fresh sausages had similar b* values to begin with but there was a sharp decrease of b* values at day 4 on DFD meat; the b* values then decreased at a slower rate for the rest of the display period.

In DFD meat, the reversion of discolouration occurred on day 4 of the display period (Figure 4.11 b). There are many reports that state that red colour and microbial growth are inversely related. Microbial growth can affect meat colour through bacterial oxygen consumption (Faustman and Cassens, 1990), so it can reduce the oxygen partial pressure to the point where metmyoglobin formation is maximized. However, further consumption of oxygen could then establish an environment permitting metmyoglobin reduction (Arihara *et al*., 1993). Butler *et al*. (1953) observed that the elevation of metmyoglobin agreed with microbial logarithmic growth but further increases in microbial growth led to the reduction of metmyoglobin. In this study, colour reversion occurred at day 4 at which point the total microbial count was 8-9 $\log_{10}$ (CFU/g) (Figure 4.9); microbial growth was followed by an obvious increase in pH at day 4 (Figure 4.8).

On the other hand, the cell counts on normal fresh sausages reached 8-9 $\log_{10}$ (CFU/g) at the end of display period (day 10); therefore, colour reversion was not observed in normal fresh sausages Arihara *et al*. (1993) further stated that only aerobic bacteria have the ability to significantly reduce the oxygen tension at the meat surface and that facultative bacteria such lactobacilli or *B. thermosphacta* might not cause this colour reversion (Kropf *et al.*, 1986).

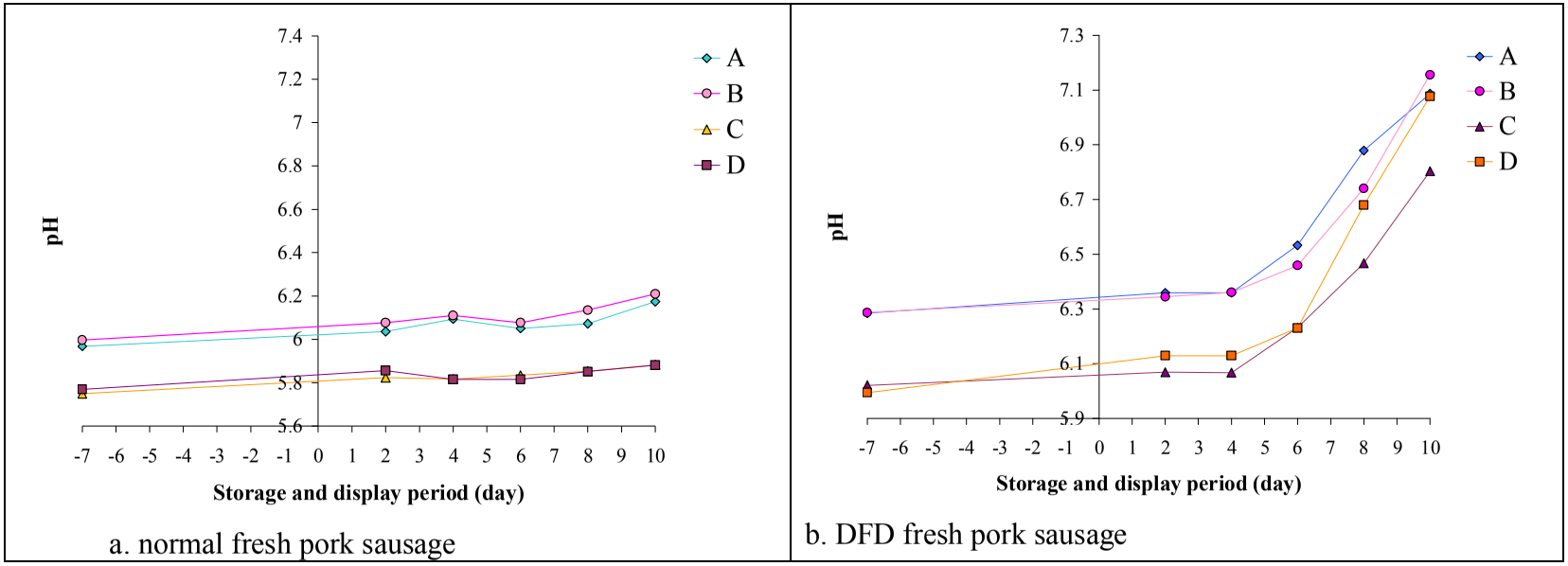
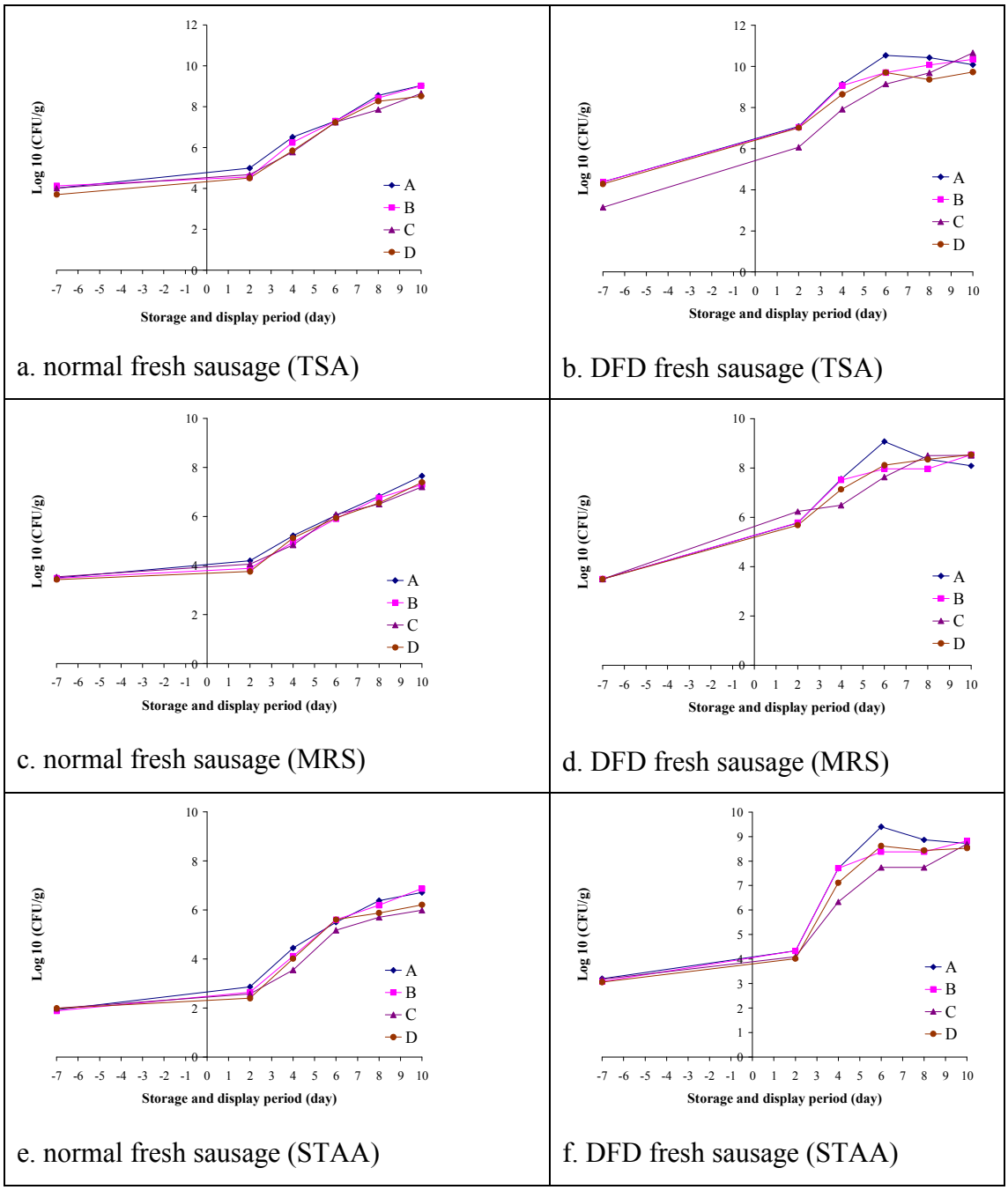


Figure 4.8: The pH profile during storage at -1°C and display at 4°C (illuminated with 850 lux – 1100 lux light) for a: normal fresh sausages and b: DFD fresh sausages. A = 0% sodium erythorbate and 0% lemon juice powder, B = 0.05% sodium erythorbate, C = 0.25% lemon juice powder, D = 0.05% sodium erythorbate and 0.25% lemon juice powder



a. normal fresh sausage (TSA)

b. DFD fresh sausage (TSA)

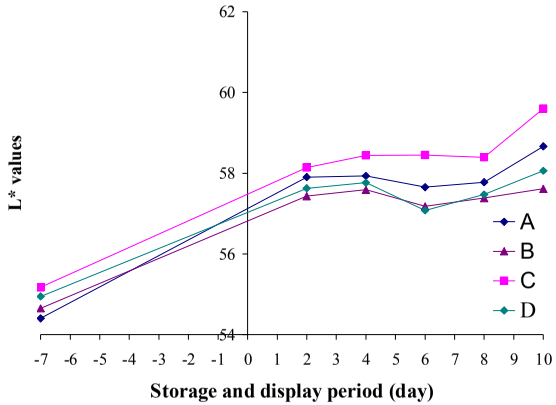
c. normal fresh sausage (MRS)

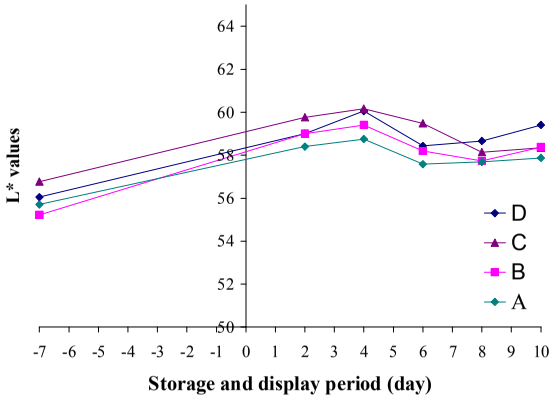
d. DFD fresh sausage (MRS)

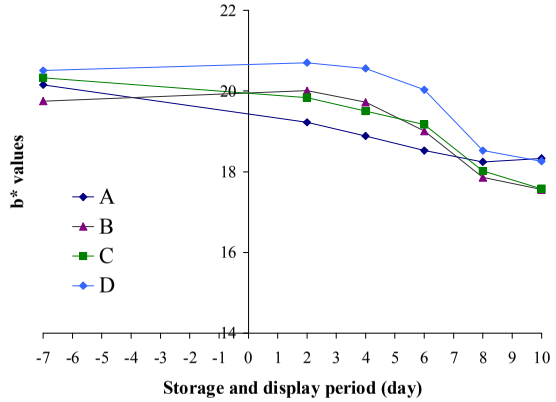
e. normal fresh sausage (STAA)

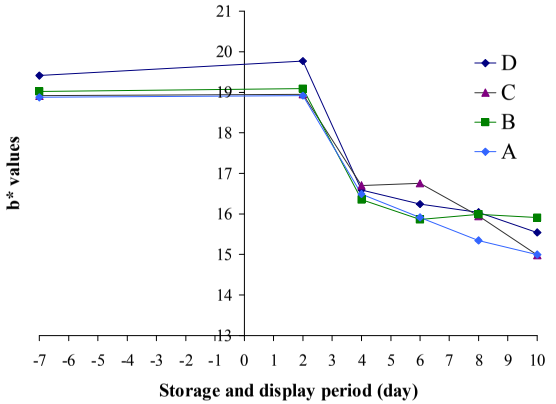
f. DFD fresh sausage (STAA)

Figure 4.9: DFD fresh sausages during storage at -1°C and display at 4°C (illuminated with 850 lux – 1100 lux light) for a: total microbial count, b: lactic acid microbial count, c: B. thermosphacta microbial count. A = 0% sodium erythorbate and 0% lemon juice powder, B = 0.05% sodium erythorbate, C = 0.25% lemon juice powder, D = 0.05% sodium erythorbate and 0.25% lemon juice powder





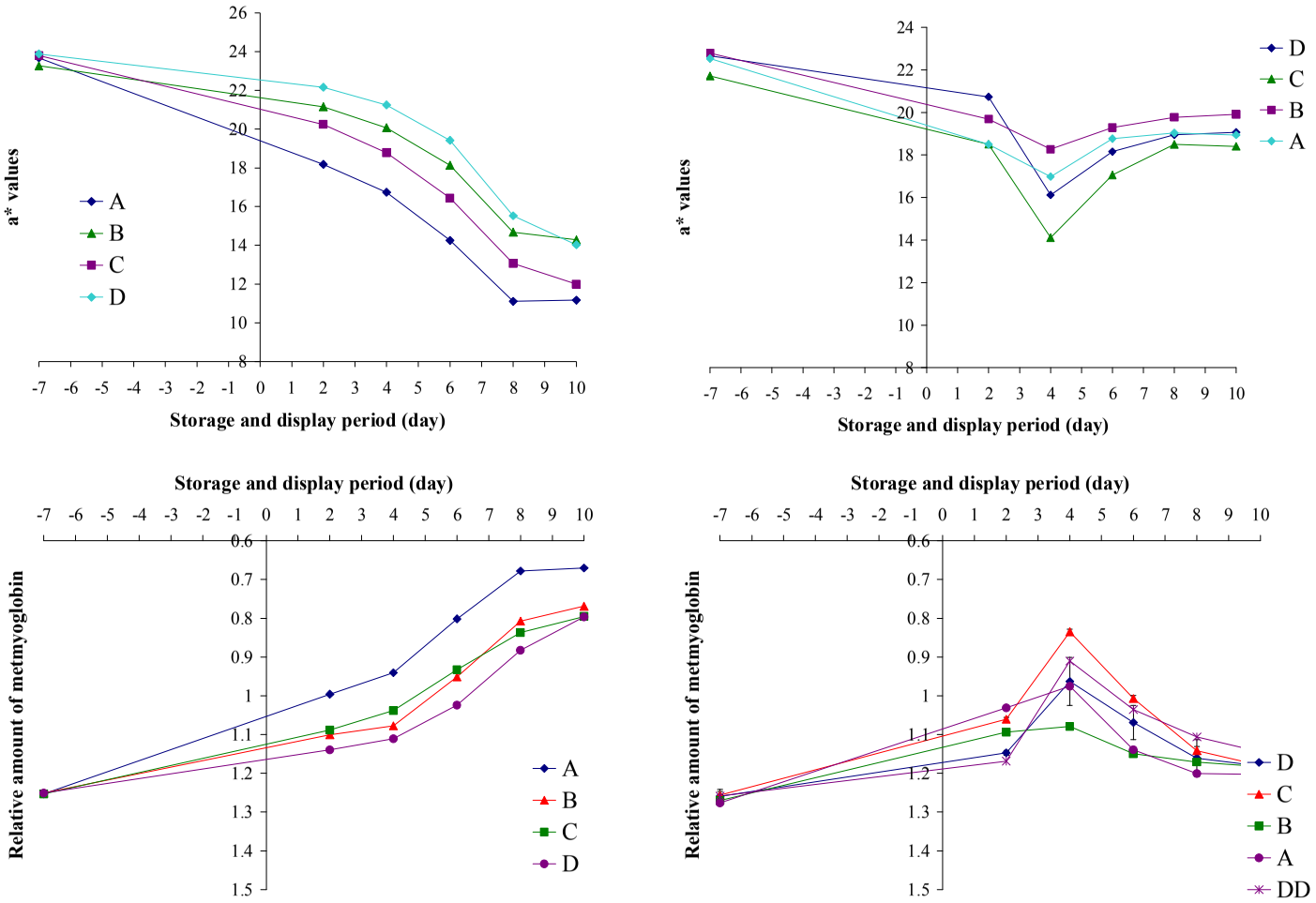




.a. Normal fresh pork sausages b. DFD fresh pork sausages

Figure 4.10: L* values and b* values for a: normal fresh sausages and b: DFD fresh sausages during storage at -1°C and display at 4°C (illuminated with 850 lux – 1100 lux light). A = 0% sodium erythorbate and 0% lemon juice powder, B = 0.05% sodium erythorbate, C = 0.25% lemon juice powder, D = 0.05% sodium erythorbate and 0.25% lemon juice powder

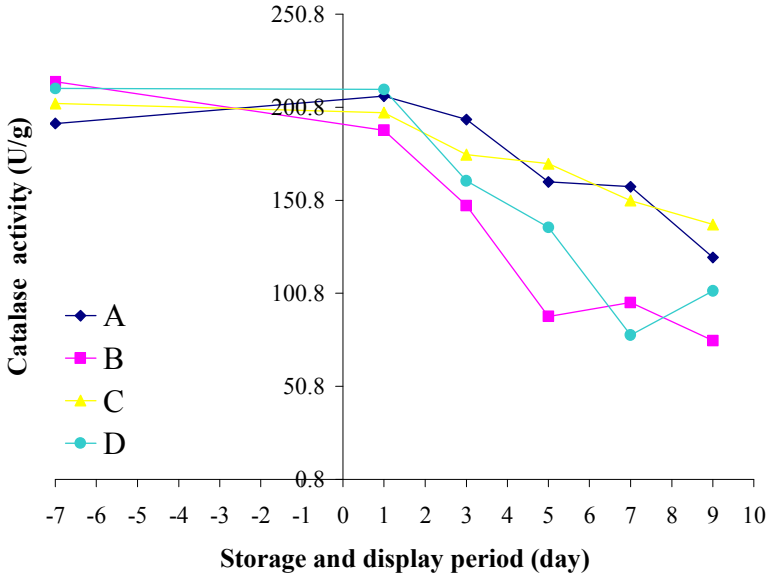
Moreover, it has been reported that *Lactobacillus fermentum* JMC1173 can produce nitric oxide in media with no nitric oxide added (Lücke, 1985); and this intermediate converted metmyoglobin to a more desirable derivative, hexacoordinate nitric oxide complex of Fe (II) myoglobin (Morita *et al*., 1998). However, this phenomena was not observed in this study so further study should be performed in the future. For antioxidant analyses, the most obvious difference in sausage formulations was for the parameter, catalase activity. Figure 4.12 shows that there was a plateau in catalase activity that started on day 3 to day 7, followed by a significant drop the following day. There is no publication that reports the relation of colour reversion and antioxidant activity. Further investigation is necessary in order to clarify the full mechanisms on how antioxidant activity affects colour reversion.



a. Normal fresh pork sausage b. DFD fresh pork sausages

Figure 4.11: a * values and relative amount of metmyoglobin for a: normal fresh sausages and b: DFD fresh sausages during storage at -1°C and display at 4°C (illuminated with 850 lux – 1100 lux light). A = 0% sodium erythorbate and 0% lemon juice powder, B = 0.05% sodium erythorbate, C = 0.25% lemon juice powder, D = 0.05% sodium erythorbate and 0.25% lemon juice powder

a. Nornal fresh sausages



b. DFD fresh sausages

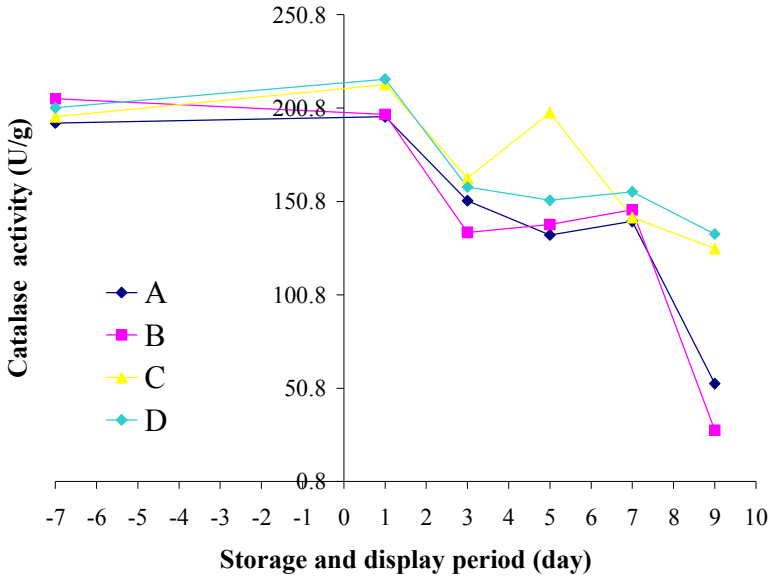


Figure 4.12: Catalase activity for a:normal fresh sausages and b: DFD fresh sausages during storage at -1°C and display at 4°C (illuminated with 850 lux – 1100 lux light. A = 0% sodium erythorbate and 0% lemon juice powder, B = 0.05% sodium erythorbate, C = 0.25% lemon juice powder, D = 0.05% sodium erythorbate and 0.25% lemon juice powder

4.2.6 The effect of retail light on antioxidant activity, colour and microbial activity

In this study, the illumination of fluorescent light with intensity of 850 – 1100 lux did not affect the colour profile, antioxidant activity and microbial activity of the fresh sausages (Table 4.17). Likewise, Ramsbottom *et al*. (1951) found that fluorescent lighting at 600 to 2000 lux intensity had no effect on colour during 3-day display. In their study, steaks stored in the dark for 3, 5 or 7 days prior to display had a display life similar to those placed in display without any prior dark storage time. The intensity of the light that is used for display is increased; there is a possibility that it can affect more damage in meat colour. However, it has been reported by Ramsbottom *et al*. (1951) found that fluorescent lighting at 600 to 2000 lux intensity has no effect on colour during 3-day display.

Display period and storage period are often used interchangeably but they don't actually have the same definition. Display period is the period when the products are offered under lighting in the refrigerated retail display. Storage period is when stored products are held in the dark under refrigeration and usually not for sale. In this study, the regular fresh sausages were stored for 7 days at a constant -1°C in the dark and then they were displayed at a constant 4°C for 10 days under constant 850 – 1100 lux illumination. In order to determine the effect of the illumination on the antioxidant capacity, colour and microbial activity of fresh sausages, formulation D sausages (0.05% sodium erythorbate and 0.25% citric acid) under normal display condition were

compared to formulation DD fresh sausages under dark display conditions at 4°C for 10 days.

It has been reported that display lighting could affect the rate of discolouration due to the temperature elevation at the meat surface (Gould, 1963) and also due to photochemical changes (Bertelsen and Skibsted**,** 1987).The temperature of the meat surface has been shown to increase proportionally with increased light intensity under both incandescent and deluxe cool white fluorescent lights (Calkins *et al.,* 1986). Higher temperatures at the meat surface speed up deteriorative influences on meat color such as oxidation and microbial metabolism. Moreover, photochemical effects are caused by certain wavelength energies that excite one or more molecules and initiate or catalyze such reactions as oxidation which leads to a change in the meat pigment, myoglobin, causing discoloration.

It has been reported that microbial growth appeared to be enhanced by illumination with fluorescent light (Djenane *et al*., 2001). Moreover, photochemical effects are caused by certain wavelength energies that excite one or more molecules and initiate or catalyze such reactions as oxidation which leads to a change in the meat pigment, myoglobin, causing discoloration. In this study, none of the light-related factors significantly affected the colour profile, antioxidant activity and microbial activity. Furthermore, product illumination also did not affect the pH of the fresh sausages during the storage and display period (Figure 4.13). Lastly, further study must be done in order to analyze the effect of light intetnsity during display period and endogeneous antioxidant activity since there is a no publication has been published regarding this.

Table 4.17: The effect of light on antioxidant activity, colour and microbial activity in fresh sausages during a ten day display period at 4°C (three batches)

Variables	SEM	*p* values treatment
Catalase activity (U/g meat)	15.30	0.1491
GSHx activity (U/g meat)	0.0710	0.7187
SOD activity (IU/g meat)	3.038	0.4955
Relative amount of Metmyoglobin	0.0650	0.5093
L* values	0.5090	0.5341
a* values	1.012	0.0804
b* values	0.7000	0.5294
Chroma	1.116	0.4651
Hue	1.694	0.5386
Total microbial count (log_{10} (CFU)/g)	0.5770	0.8195
B. thermosphacta microbial count (log_{10} (CFU)/g)	0.6510	0.9563
Lactic acid microbial count (log_{10} (CFU)/g)	0.4170	0.7680

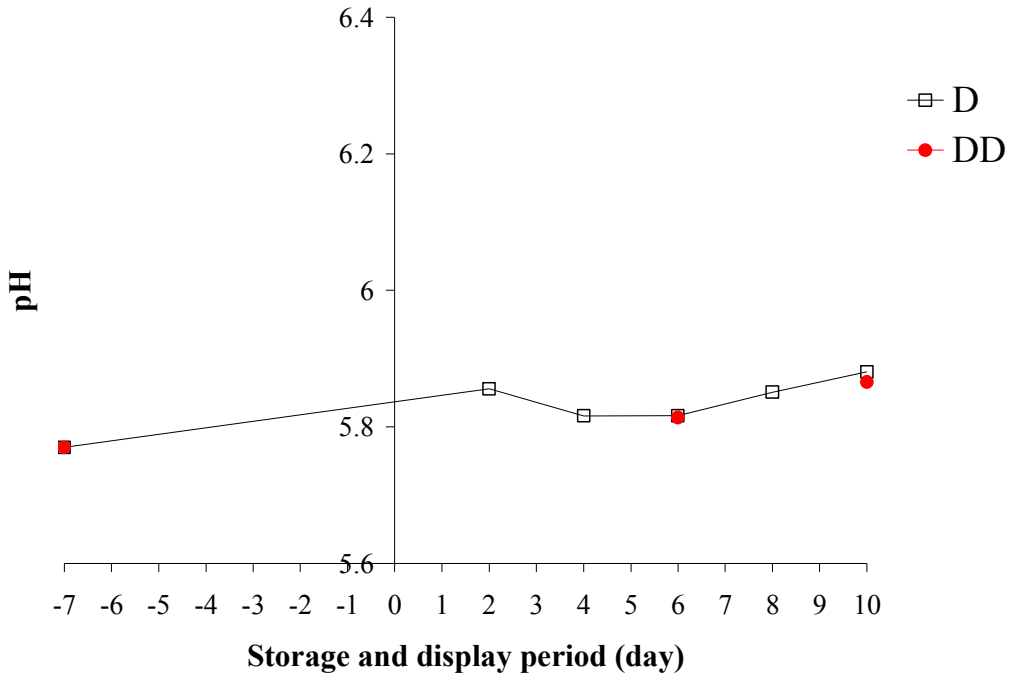


Figure 4.13: pH of formulation D fresh sausages that were displayed under 850 – 1100 lux illumination and formulation DD fresh sausages that were displayed in the dark at 4°C. D = 0.05% sodium erythorbate and 0.25% lemon juice powder, DD = D that was stored under dark during display period for 10 days at 4°C

5.0 Conclusions

Fresh sausage is usually sold raw and with a minimum of 7.5% meat protein and 9% total protein. In this study, fresh sausages were made from pork picnic shoulder as the meat ingredient and water/ice (12%, w/w), salt (1.5%, w/w), lemon juice powder (0.25%, w/w), and sodium erythorbate 0.05% (*i.e.*, 500 ppm) as the non-meat ingredients. The colour stability during the distribution and display period is very important because unstable meat colour ultimately means shorter shelf-life due to consumer buying habits.

It was shown in the first study that both catalase and superoxide dismutase were responsible for patty discolouration, as indicated by significant ($p<0.05$) decreases in a* values and relative amount of metmyoglobin during the distribution and display period. In this study, ground pork became discoloured more rapidly than fresh sausages, a phenomenon speculated to be due to more effective catalase and superoxide dismutase activities in fresh sausages. Moreover, the total microbial count, which include lactic acid bacteria and *B. thermosphacta,* increased significantly over time ($p<0.05$) while the redox potential decreased for both ground pork and fresh sausages.

In the second study, sodium erythorbate was found to significantly interact with catalase and a* values (p>0.05). Thus, it can be concluded that 0.05% sodium erythorbate was responsible for colour stability during distribution and display period of fresh sausages. Sodium erythorbate lowered the redox potential of the fresh sausages, improving the functionality of metmyoglobin reductase with the end result being improved colour over the distribution and display period. There was no synergistic effect found between 0.25% lemon juice powder and 0.05% sodium erythorbate in this study. From these results, it can be concluded that it is more beneficial to only add 0.05% sodium erythorbate to prolong shelf life in fresh sausages.

6.0 Future work

In this study, there was no synergistic interaction between lemon juice powder and sodium erythorbate at level 0.25% and 0.05%, respectively, the amount that is usually used in the industry. The concentration of the citric acid in fresh sausage patty which contains 0.25% lemon juice powder was only 0.066%. This amount might be too low to cause a significant effect with sodium erythorbate. Therefore, in the future, a range of levels of citric acid may be analyzed to find out the optimum concentration of citric acid that could give a significant synergistic effect with sodium erythorbate. From this concentration of citric acid, the amount of lemon juice powder can then be derived and added into the formulation. Moreover, it might be better to add citric acid directly than to add lemon juice powder.

Further investigation is also necessary in order to clarify the full mechanisms of how antioxidant activity, especially catalase activity, affects colour reversion on DFD fresh sausage because the activity of antioxidants are different at different pH as in DFD fresh pork sausage.Furthermore, it would be beneficial to know what factors could affect the *in situ* levels of reductants in the meat environement because the reducing environment could control rates of metmyoglobin reduction to oxymyoglobin.

Lastly, the effect of metmyoglobin reductase during storage on colour, microbial and redox potential should be investigated in more detail. This enzyme might have an effect on overall endogenous antioxidant that then could affect the shelf life of fresh pork sausage.

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Appendix 1: Buffer recipes

Phosphate Buffer 50 mM pH 7.4

50 mM KH_2PO_4 (MW 136.09 g/mole) = 6.805 g in 1000 mL
50 mM K_2HPO_4 (MW 174.18 g/mole) = 8.709 g in 1000 mL

pH 7.4	100 mL	300 mL	1000 mL
$H_2PO_4^-$	20.1	60.2	200.8
$HPO4^{2-}$	79.9	239.8	799.2

TRIS-Cacodylic acid 50mM pH 8.2 (+ 1mM DETPA)

50 mM TRIS base (MW121.14 g/mole) = 3.0285 g in 500 mL
1 mM DETPA (MW 393.35 g/mole) = 0.1967 g in 500 mL

Adjust pH to 8.2 with 1M cacodylic acid (MW 138 g/mole) = 0.69 g in 5 mL

EDTA 16 mM in Phosphate Buffer 400 mM pH 7.4

400 mM KH_2PO_4 (MW 136.09 g/mole) = 54.436 g in 1000 mL
400 mM K_2HPO_4 (MW 174.18 g/mole) = 69.672 g in 1000 mL

pH 8.4	100 mL	300 mL	1000 mL
$H_2PO_4^-$	20.1	60.2	200.8
HPO_4^{2-}	79.9	239.8	799.2

16 mM EDTA (MW 292.25 g/mole) = 4.676 g in 1000 mL

PBS 1x (10 mM) pH 7.4

0.26 g KH_2PO_4 + 2.17 g Na_2HPO_4 $7H_2O$ + 8.71 g NaCl in 800 ml dH_2O

Adjust pH to 7.4 and bring volume to 1 L with distilled water

Appendix 2: Microbiological media recipes

Tryptic Soy Agar (TSA) + 0.1% yeast extract

0.5 g yeast extract + 15 g Tryptic Soy Broth + 7.5 g agar in 500 mL distilled water

De Man Ragosa and Sharpe Agar (MRS)

27.5 g MRS + 7.5 g agar in 500 mL distilled water

Streptomycin Thallous Acetate Actidione (STAA)

18.5 g STAA agar base + 7.5 g glycerol + 1 vial STAA supplement in 500 mL distilled water

STAA supplement: 250 mg streptomycin sulphate, 25 mg thallous acetate, 25 mg cycloheximide

Appendix 3: TEAC analysis calculation

TEAC is defined as the concentration *(mmol/L)* of Trolox having the equivalent antioxidant capacity of a 1.0 mmol/L solution of the substance under investigation (Miller *et al*., 1993).

Trolox stock 2 mM ➔ 50 mg in 100 mL PBS 5 mM pH 7.4

Trolox concentration that was used for the construction of Trolox standard curve

Trolox stock 2 mM Volume (uL)	In PBS (uL)	Trolox concentration (mM)
0	1000	0
200	800	0.4
400	600	0.8
600	400	1.2
800	200	1.6
1000	0	2

Spectrophotometer set up at 734 nm, 30°C (blank: 5 mM PBS pH 7.4)

Assay: 20 μL Trolox + 2 mL (ABTS + potassium persulfate) ➔ **100x dilution**

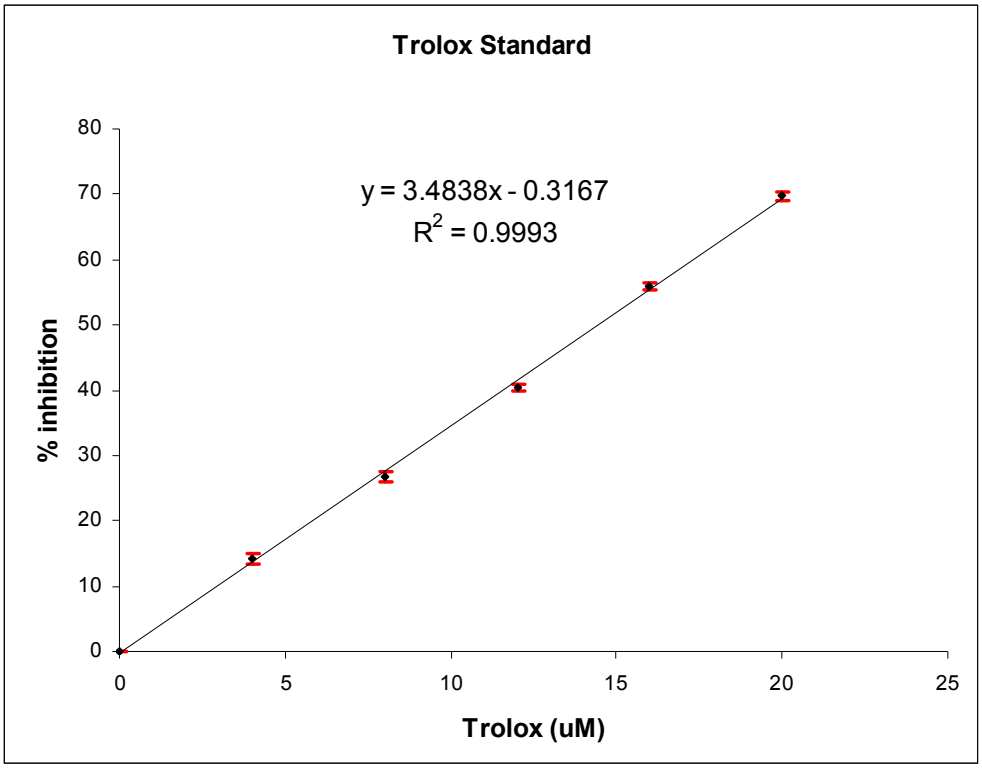
Concentration (mM)	Abs	Average	Standard Deviation	Coefficient Variation (%)	Final Concentration (μM) in cuveettee	% inhibition
0	0.67958					
0	0.68545	0.68252	0.00415	0.61%	0	0.00%
0.4	0.58149					
0.4	0.59021	0.58585	0.00617	1.05%	4	14.16%
0.8	0.50301					
0.8	0.49562	0.49932	0.00523	1.05%	8	26.84%
1.2	0.40884					
1.2	0.40381	0.40633	0.00356	0.88%	12	40.47%
1.6	0.29893					
1.6	0.3038	0.30137	0.00344	1.14%	16	55.84%
2	0.20917					
2	0.20289	0.20603	0.00444	2.16%	20	69.81%

Calculation:

$$\% \text{ inhibition} = \frac{Abs_{734} \text{ (blank – standard or sample) at 6 min}}{Abs_{734} \text{ (blank) at 6 min}} \times 100\%$$

$$= [(0.68252-0.58585)/ 0.68252] \times 100\% = 14.16\%$$

Trolox Standard



Sample	Abs	% inhibition	Trolox (µM)
0	0.68252	0.00%	
Meat extract	0.22328	67.29%	19.404788
Meat extract 1:2	0.42254	38.09%	11.024582

Calculation:

y = 3.4838x-0.316

y =38.09% ➔ **x** = (y + 0.3167)/3.4838
= 11.024582 µM Trolox

2x dilution ➔ 22.049164 µM Trolox

22.049164 μM Trolox = 22.049164 nmoles Trolox/mL

x 2.02 mL

44.539311 nmoles Trolox in cuvettee (20 μL meat extract)

Volume of meat extract after extraction process with 5 g meat sample = 20.5 mL

The total antioxidant capacity in meat sample

= [(20500 μL /20 μL)] * 44.539311 nmoles Trolox

= 45.65 μmoles Trolox/5 g

= 9.130 μmoles Trolox/g

Appendix 4: SOD calculation

One unit of SOD is defined as the activity that inhibits the reaction by *50%* (Renerre *et al.*, 1996; Gatellier *et al.*, 2004).

Spectrophotometer set up at 340 nm, room temperature (blank: Tris-Cacodilyc acid buffer (50 mM, pH 8.2)

1.9 mL Tris-Cacodylic acid buffer (50 mM, pH 8.2) and 50 µL pyrrogallol (10 mM) were added into each of the following meat extract and phosphate buffer to make up the sample standard.

Meat extracts dilution to establish sample standard

Meat extract (µL)	50 mM phosphate buffer pH 7.4 (µL)
70	0
60	10
50	20
40	30
30	40

Sample standard (meat extract vs. %inhibition)

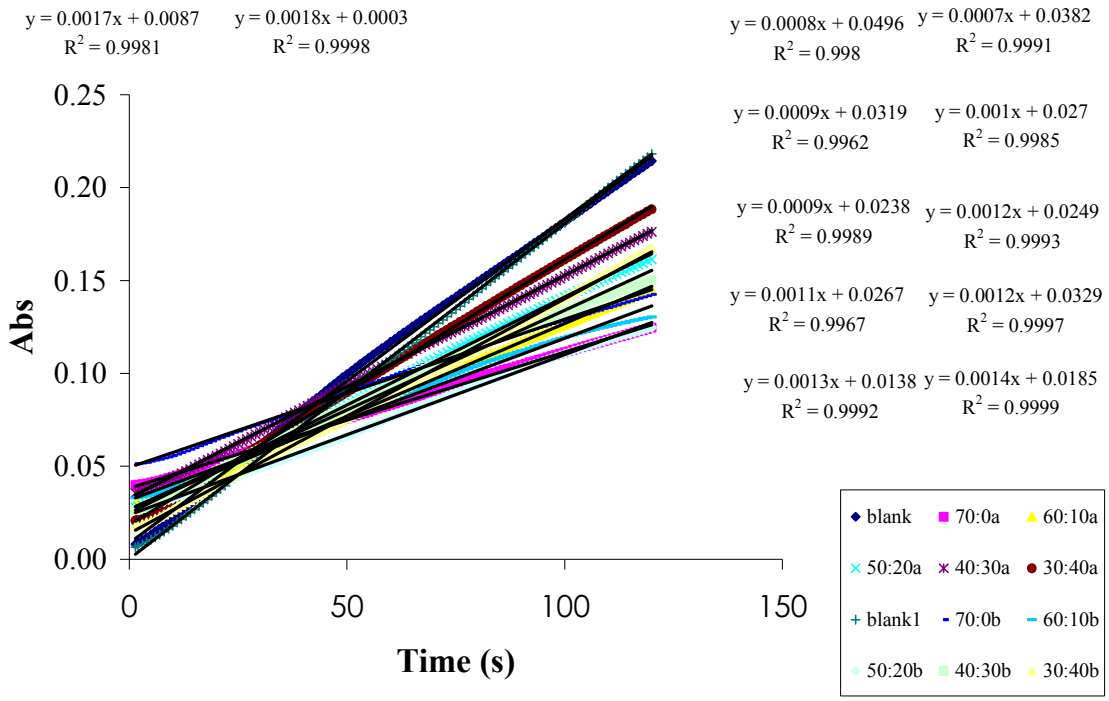
Meat extract	Slope a	Slope b	Average Slope	% inhibition
Blank	0.0017	0.0018	0.00175	0.00
70	0.0008	0.0007	0.00075	57.14
60	0.0009	0.0010	0.00095	45.71
50	0.0009	0.0012	0.00105	40.00
40	0.0011	0.0012	0.00115	34.29
30	0.0013	0.0014	0.00135	22.86
0				0.00

Calculation:

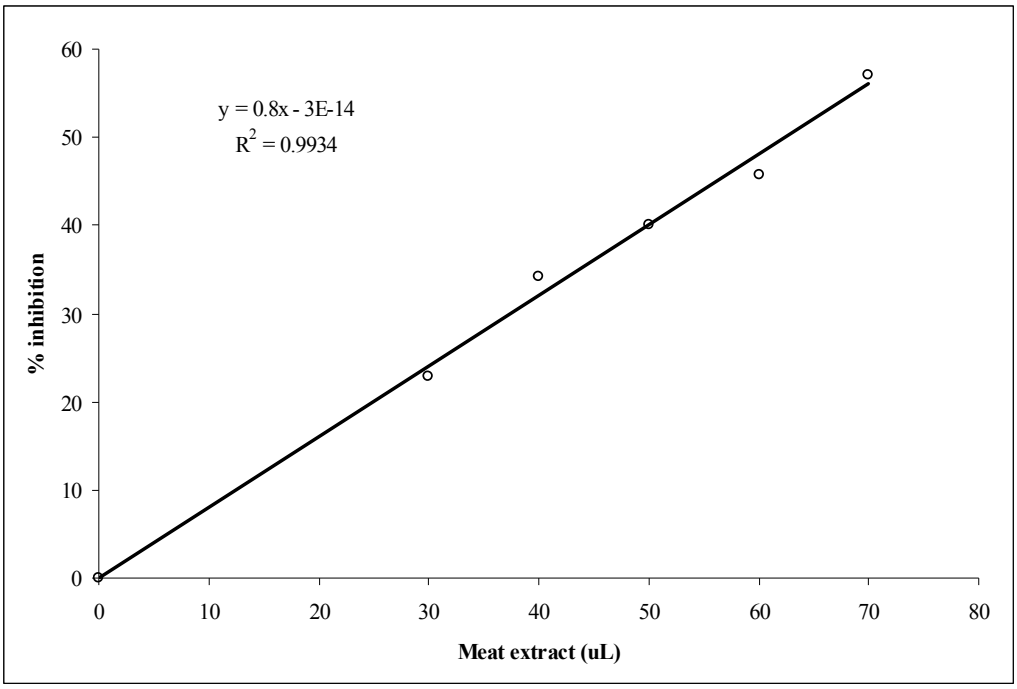
$$\% \text{ inhibition} = \frac{(\text{slope of blank} - \text{slope of meat extract})}{\text{slope of blank}} \times 100\%$$

$$= [(0.0017-0.00075)/0.0017] * 100\% = 57.14\%$$

Graph showing the increase in absorbance of pyrogallol at 340 nm during the first 2 min (in duplicate) at room temperature



Sample standard



Calculation:

y = 0.8x – 0.014

when y = 50% ➔ **x** = (y + 0.014)/ 0.8

x = 62.51 µL of meat extract = 1 U

Volume of meat extract after meat extraction of 5 g meat sample = 20.5 mL

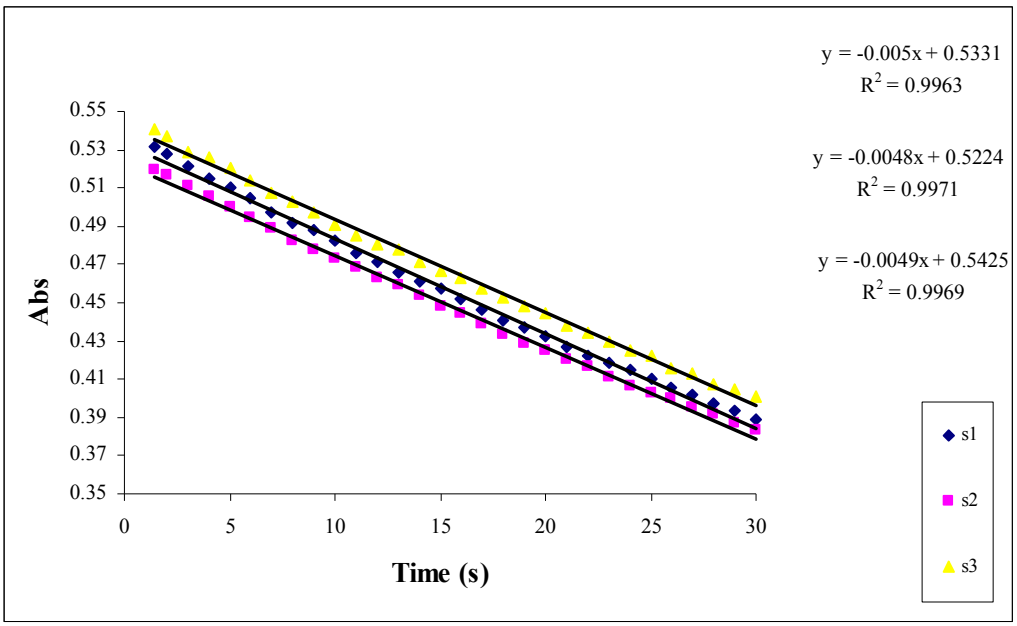
The SOD activity in meat sample = (20500 µL/62.51 µL)/5 g

= 65.59 U/g

Appendix 5: Catalase calculation

One unit of catalase activity is defined as the quantity of meat extract needed to decompose 1 µmol of H_2O_2/min (Hernández et al., 2002).

The decrease in H_2O_2 absorbance at 240 nm at room temperature during the first 30 secs for 1 sec interval



Calculation:

Average slope= 0.00413/sec= 0.248/min

H_2O_2 ϵ_{240} = 43.6/M cm in 1 cm cuvettee ➔ 43.6/M

1 M ➔ 43.6

x M ➔ 0.248

x = (0.248/43.6) * 1 M = 5.688073 mM/min = 5.688073 μmoles/mL min

* 3.0 mL

17.06422 μmoles of H_2O_2/min

In the cuvettee:

1.0 mL of meat extract (diluted 1:8 with 50 mM phosphate buffer pH 7.4)

1.5 mL 50 mM potassium phosphate buffer (pH 7.4)

0.5 mL 100 mM H_2O_2

Meat extract (1:8) = 1000 μL ➔ 17.06422 μmoles of H_2O_2/min

x μL ➔ 1 μmoles of H_2O_2/min

x = 58.60215 μL = 0.058 mL = 1 U

The volume of meat extract after meat extraction of 5 g meat sample = 20.5 mL

The activity of catalase in meat sample = (20.5 mL/ 0.058 mL)/ 5 g

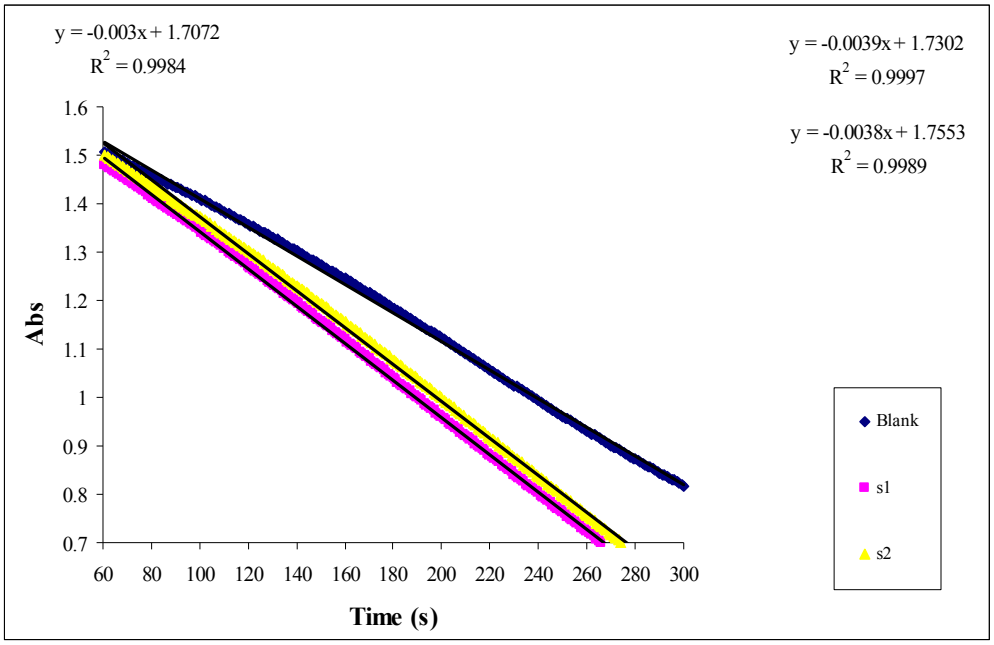
= 70.69 U/g

Dilution 8x ➔ 565.5172 U/g

Appendix 6: Glutathione peroxidase calculation

GSHPx activity is the amount of extract that is required to oxidize *1µmole NADPH/min* at 37°C (Hernandez *et al*., 2004).

The decrease in NADPH absorbance at 340 nm at 37°C that was taken 1 min after the reaction was initiated via the addition of *tert*-butyl hydroperoxide, TBHP



Calculation:

Average slope = 0.00385/sec

Blank = 0.00300/sec

= 0.00085/sec = 0.051/min

NADPH ϵ_{340} = 6300/M cm in 1 cm cuvettee ➔ 6300/M

1 M ➔ 6300

x M ➔ 0.051

x = (0.051/6330) *1 M = 8.056 μM/min = 8.056 nmoles/mL min

*1.95 mL

15.71 nmoles/min

The reaction medium in the cuvette for GSHPx determinations

	Volume (μL)
16 mM EDTA in 400 mM phosphate buffer (pH 7.4)	500
Glutathione reductase 15 U/mL	50
60 mM Glutathione reduced (GSH)	200
50 mM phosphate buffer (pH 7.4) or meat extract	100
Deionized water	400
Incubation for 10 min at 37°C	
3 mM NADPH in 0.1% (w/w) $NaHCO_3$	200
6.3 mM *tert*-butyl hydroperoxide (TBHP)	500

Meat extract 100 μL ➔ 15.71 nmoles of NADPH/min

x μL ➔ 1000 nmoles of NADPH/min

x = 6365.37 μL meat extract to decompose 1 = 6.365 mL = 1 U

The volume of meat extract after meat extraction of 5 g meat sample = 20.5 mL

The activity of GSHPx in meat sample = (20.5 mL/ 6.365 mL)/ 5 g

= 0.644 U/g